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(54) **Title:** T7 PROMOTER–BASED EXPRESSION SYSTEM

(57) **Abstract**

An improved T7 based promoter–driven protein expression system comprising an operator sequence downstream of the T7 promoter sequence, and having a further operator sequence upstream of the T7 promoter sequence.

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T7 PROMOTER–BASED EXPRESSION SYSTEM

The invention relates to expression systems for the recombinant synthesis of polypeptides, in particular to T7 promoter-driven protein expression systems. The invention also relates to expression vectors for use in such systems.

A large number of mammalian, yeast and bacterial host expression systems are known (Methods in Enzymology (1990), 185, Editor: D.V. Goeddel). Of particular interest are those which use T7 RNA polymerase. The ability of T7 RNA polymerase and equivalent RNA polymerases from T7-like phages to transcribe selectively any DNA that is linked to an appropriate promoter can serve as the basis for a very specific and efficient production of desired RNAs both *in vitro* and inside a cell.

US-A-4952496 (Studier) discloses a process whereby T7 RNA polymerase can be expressed and used to direct the production of specific proteins, all within a host *E. coli* cell. Specific proteins of interest include antigens for vaccines, hormones, enzymes, or other proteins of medical or commercial value. Potentially, the selectivity and efficiency of the phage RNA polymerase could make such production very efficient. Furthermore, the unique properties of these phage RNA polymerases may make it possible for them to direct efficient expression of genes that are expressed only inefficiently or not at all by other RNA polymerases. These phage polymerases have the further advantage that it is possible to selectively inhibit the host cell RNA polymerase so that all transcription in the cell will be due to the phage RNA polymerase.

An expression system based on the above is now commercially available. This is the pET system obtainable from Novagen Inc. 597 Science Drive, Madison, WI 53711. This system is suitable for the cloning and expression of recombinant proteins in *E. coli*. See also Moffat et al, J.Mol. Biol., 1986, 189, 113-130; Rosenberg et al, Gene, 56, 125-135; and Studier et al, Meth. Enzymol. 1990, 185, 60-89.

However, despite the provision of the pET system, there remains the need for further, improved T7 promoter-driven expression systems.

We have now devised such a system which provides improved control of expression and improved levels of protein expression, when compared to available T7-based expression systems. We provide a T7 promoter-driven expression system wherein basal expression in the absence of inducer is reduced to a level which permits the cloning and expression of toxic gene products not possible with currently available T7 based expression systems whilst not influencing induced productivity. Moreover, our present invention also allows control of production of heterologous proteins in an inducer concentration-dependent manner over a wide range of expression levels so

that an optimum level of expression can be identified. This level of control over expression and production of heterologous protein is not possible with currently available T7 based expression systems.

Therefore in a first aspect of the invention we provide a T7 promoter-driven protein expression system comprising an operator sequence downstream of the T7 promoter sequence, and having a further operator sequence upstream of the T7 promoter sequence.

We have found that the further operator is preferably a native lac operator (lacO) sequence. Or a perfect palindrome operator (ppop) sequence. More preferably the native (lac) operator sequence downstream of the T7 promoter sequence is replaced by a ppop sequence, so as to provide a tandem ppop operator.

The T7 promoter driven expression system is conveniently constructed as follows: The target gene of interest is cloned in a plasmid under control of bacteriophage transcription and translation signals. The target gene is initially cloned using a host such as *E.coli* DH5α, HB101 that does not contain the T7 RNA polymerase gene. Once established, plasmids are transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under for example lac UV5 control. Other convenient promoters include lac, trp, tac, trc, and bacteriophage λ promoters such as pL and pR. Expression is then induced by the addition of an inducer such as IPTG (isopropyl-β-D-1-thiogalactopyranoside), lactose or melibiose. Other inducers may be used and are described more fully elsewhere. See The Operon, eds Miller and Reznikoff (1978). Inducers may be used individually or in combination.

The plasmid preferably includes one or more of the following: a selectable antibiotic resistance sequence, a cer stability element, and a multiple cloning site. The construction of appropriate plasmids will be apparent to the scientist of ordinary skill. Examples of preferred plasmids comprising one or more of the above features are illustrated by the pZT7#3-series of plasmids in the accompanying Figures. These were constructed starting from a vector pZEN0042 disclosed (as pICI0042) in our European Patent Application No. 0 502 637 (ICI). The 3-series plasmids of this invention include pZT7#3.0, pZT7#3.1, pZT7#3.2 and pZT7#3.3. A particularly preferred plasmid of this invention is the pZT7#3.3 plasmid.

The chromosomal copy of the T7 RNA polymerase gene, for example under lac UV5 control, is preferably introduced into the host cells via the λ bacteriophage construct, λDE3, obtainable from Novagen. The T7 RNA polymerase expression cassette may also be delivered to the cell by infection with a specialised bacteriophage λ transducing phage that carries the gene (CE6, see US-A-4952496).

Compatible plasmids such as pLysS and pLysE (also available from Novagen) may also be introduced into the expression host. These plasmids encode T7 lysozyme, which is a natural and selective inhibitor of T7 RNA polymerase, and thus reduces its ability to transcribe target genes in uninduced cells. pLysS hosts produce low amounts of T7 lysozyme, while pLysE hosts produce much more enzyme and therefore provide more stringent control.

Any convenient compatible prokaryotic or eukaryotic host cell may be used. The most commonly used prokaryotic hosts are strains of *E.coli*, although other prokaryotic hosts such as *Salmonella typhimurium, Serratia marsescens, Bacillus subtilis* or *Pseudomonas aeruginosa* may also be used. Mammalian (e.g. Chinese hamster ovary cells) or other eukaryotic host cells such as those of yeast (e.g. *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Schizosaccharomyces pombe* or *Kluyveromyces lactis*), filamentous fungi, plant, insect, amphibian or ovarian species may also be useful. A particular host organism is a bacterium, preferably *E. coli* (e.g. K12 or B strains).

Any convenient growth medium may be used depending on the host organism used. For *E.coli*, practice of this invention includes, but is not limited to complex growth media such as L-broth or minimal growth media such as M9 (described hereinafter).

The invention will now be illustrated but not limited by reference to the following detailed description Examples, Tables and Figures wherein:

Table 1 gives details of plasmids expressing h-TNFα used in the Examples.

Tables 2-4 gives details of vectors used in the Examples and their relative performance.

Table 5 gives details of the composition of M9 minimal growth medium.

Table 6 gives details of h-TNFα expression in various growth media.

Table 7 gives details of host/transformation efficiencies for vectors used in the Examples.

Table 8 gives details of DNase 1 productivity in conjunction with the pZT7#3.3:DNase 1vector.

Tables 9-11 give details of accumulation levels for LAR d1 (aa1275-1623), ZAP70 (4-260) 6HIS and MCP-1 {9-76} used in the Examples.

Table 12 shows the sequences of oligonucleotides used in the construction of pZT7#3.3 and intermediate vectors.

Table 13 shows the nucleic acid sequence of hTNFα.

Table 14 shows the ZAP70 (4-260) 6HIS nucleic acid sequence.

Table 15 shows the LAR d1 (aa1275-1623) nucleic acid sequence.

Table 16 shows the bovine pancreatic DNase 1 nucleic acid sequence.

Table 17 shows the human carboxypeptidase B (mutant D253>K) 6His cmyc sequence.

Table 18 shows various *E. coli* expression strains.

Table 19 shows the human monocyte chemotactic protein MCP-1 {9-76} sequence.

Table 20 shows the A5B7 $F(ab')_2$ nucleic acid sequence.

Figure 1 shows the pZEN0042 plasmid.

Figure 2 shows the construction of pZEN0042#a from pZEN0042.

Figure 3 shows the pZEN0042#b plasmid.

Figure 4 shows the pZEN0042#c plasmid.

Figure 5 shows the pZEN0042#d plasmid.

Figure 6 shows the pZEN0042#e plasmid.

Figure 7 shows the pZT7#2.0 plasmid.

Figure 8 shows the pZT7#2.1 plasmid.

Figure 9 shows the pZT7#3.0 plasmid of the invention.

Figure 10 shows the pZT7#3.2 plasmid of the invention.

Figure 11 shows the pZT7#3.1 plasmid of the invention.

Figure 12 shows the pZT7#3.3 plasmid of the invention.

Figure 13 shows the peak specific productivity attained at various IPTG concentrations.

Figure 14 shows the maximum hTNFα accumulation level attained at various IPTG concentrations..

Figure 15 shows the accumulation of biologically active CPB[D253K]-6His-cmyc (as µg active material /L of culture) attained at various IPTG concentrations.

Figure 16 shows the accumulation in the periplasm of *E.coli* of biologically active $A5B7(Fab')_2$/A5B7(Fab′) (as mg active material/L of culture) attained at various IPTG concentrations.

Specific description:

1. Generation of pZT7 series vectors

The starting vector for generation of pZT7#3.3 was pZEN0042, described fully in our European Patent Application, Publication No. 0502637. Briefly, this vector contains the tetA/tetR inducible tetracycline resistance sequence from plasmid RP4 and the cer stability sequence from plasmid pKS492 in a pAT153 derived background (Figure 1).

1(i) Cloning of lac I

The sequence of lac I including the lac repressor coding sequence and lac I promoter was generated by polymerase chain reaction using genomic DNA prepared from *E.coli* strain MSD 101 (W3110). Nsi I restriction endonuclease sites were generated at both ends of the sequence by incorporation into the PCR primers #1 a

nd #2 (Table 12). The PCR product obtained was digested with Nsi I and cloned into pZEN0042 at the Pst I site (Pst I and Nsi I have compatible cohesive ends resulting in both sites being destroyed). Both orientations of lac I were obtained. A clone with a correct sequence lac I in the anti-clockwise orientation was identified (=pZEN0042#a) (Figure 2).

1(ii) Cloning of polylinker

A new multiple cloning site was generated in pZEN0042#a to allow subsequent cloning of the T7 expression cassette. This was achieved by digesting pZEN0042#a with EcoR I and BamH I and ligating with annealed, synthetic oligomers #3 and #4 (Table 12).

The polylinker of the resulting vector, pZEN0042#b, had the following restriction sites: EcoRI - Nde I - Kpn I - Sca I - Spe I - BamH I - Xho I - Pst I - Hind III - Bgl II.

pZEN0042#b is shown in Figure 3.

2. Cloning of T7 expression elements

2(i) T7 terminator

The T7 terminator sequence from T7 gene 10 was cloned as annealed synthetic oligomers #5 and #6 (Table 12) between the Hind III and Bgl II sites of pZEN0042#b to generate pZEN0042#c (Figure 4).

2(ii) $tRNA^{arg5}$

The $tRNA^{arg5}$ transcriptional reporter sequence (Lopez, *et al*, (1994), NAR 22, 1186-1193, and NAR 22, 2434) was cloned as annealed synthetic oligomers #7 and #8 (Table 12) between the Xho I and Pst I sites in pZEN0042#c to generate pZEN0042#d (Figure 5).

2(iii) Upstream terminator

As the tetA sequence has no recognisable terminator, a T4 terminator sequence was cloned upstream of the EcoR I site to reduce potential transcriptional readthrough from tetA (or any other unidentified promoter sequence) into the T7 expression cassette (to be cloned

downstrem of the EcoR I site). Annealed synthetic oligomers #9 and #10 (Table 12) containing the T4 terminator sequence and an additional Nco I site were cloned between the Stu I and EcoR I sites in pZEN0042#d to generate pZEN0042#e (Figure 6).

2(iv) T7 lac promoter

The T7 promoter and lac operator sequence was cloned as annealed synthetic oligonucleotides #11 and #12 (Table 12) between the EcoR I and Nde I sites of pZEN0042#e to generate pZT7#2.0 (Figure 7). This configuration of T7 promoter and lac operator is equivalent to pET11a.

2(v) T7 promoter with perfect palindrome operator

The T7 gene 10 promoter incorporating a perfect palindrome lac operator sequence (Simons *et al* (1984), PNAS 81,1624-1628) was cloned as annealed synthetic oligomers #13 and #14 (Table 12) between the EcoR I and Nde I sites of pZEN0042#e to generate pZT7#2.1 (Figure 8).

2(vi) Upstream lac operator

A second native lac operator sequence was cloned 94 base pairs upstream of the lac operator sequence in pT7#2.0 as annealed synthetic oligomers #15 and #16 (Table 12) between the Nco I and EcoR I sites of pZT7#2.0 to generate pZT7#3.0 (Figure 9).

The same lac operator sequence was cloned similarly into pZT7#2.1 to generate pZT7#3.2 (figure 10).

2(vii) Upstream perfect palindrome lac operator

A perfect palindrome operator sequence was positioned 94 bp upstream of the lac operator sequence of pZT7#2.0 by cloning of annealed synthetic oligomers #17 and #18 (Table 12) between the Nco I and EcoR I sites of pZT7#2.0 to generate pZT7#3.1 (Figure 11).

The same perfect palindrome operator sequence was cloned similarly into pZT7#2.1 to generate pZT7#3.3 (Figure 12).

3. Generation of test constructs

pET11a

T7 expression vector pET11a was obtained from Novagen Inc and used as a control for the testing of the pZT7 vectors

3(i) hTNFα

An Nde I - BamH I DNA fragment containing the coding sequence of human TNFα was cloned between the Nde I and BamH I sites in vectors pZT7#2.0, pZT7#2.1, pZT7#3.0, pZT7#3.1, pZT7#3.2, pZT7#3.3 and pET11a. The sequence cloned is shown in Table 13

3(ii) ZAP70 (4-260) 6HIS

A DNA fragment encoding amino acids 4-260 of human protein tyrosine kinase ZAP70 with a C-terminal hexahistidine tag sequence was subcloned as a Nde I - Bgl II fragment between the Nde I and BamH I cloning sites of pET11a and pZT7#3.3. The sequence cloned is shown in Table 14.

3(iii) LAR d1 (aa1275-1623)

The leukocyte antigen related protein tyrosine phosphatase domain 1 (aa1275-1613) was subcloned as a Nde I - Bgl II fragment into pET 11a and pZT7#3.3 (Nde I - BamH I). The sequence cloned is shown in Table 15.

3(iv) human carboxypeptidase B (mutant D253>K) 6His cmyc

The coding sequence of a mutant human carboxypeptidase (D253>K) with a C-terminal hexahistidine cmyc tag sequence was placed downstream of the *Erwinia carotovora pel B* secretory leader sequence and cloned into pZT7#3.3 and pET11a. The sequence cloned is shown in Table 17

3(v) Bovine pancreatic DNase 1

The coding sequence of bovine pancreatic DNase I was cloned as an Nde I - Bgl II fragment between the Nde I and BamH I sites in pZT7#3.3. The sequence cloned is shown in Table 16.

This sequence could not be cloned without mutation in pET11a.

However, a bovine pancreatic DNase1 sequence had previously been cloned into pET11 (not pET11a) (Doherty *et al* (1993) Gene, 136, pp337-340). In this construct the 5' untranslated region including the ribosome binding site is derived from the native bovine pancreatic DNase 1 sequence rather than from T7 gene 10 as in pT7#3.3. This construct, pAD10, was used as a control for pT7#3.3.

3(vi) human monocyte chemotactic protein MCP-1 {aa9-76}

The coding sequence of human monocyte chemotactic protein (aa9-76) was cloned between the Nde I and BamH I sites of pZT7#3.3 and pET11a. The sequence cloned is shown in Table 19.

3(vii) A5B7 F(ab')$_2$

The coding sequence of A5B7 F(ab')$_2$ was placed downstream of the *Erwinia carotovora pel B* secretory leader sequence and cloned between the Nde I and BamH I sites of pZT7#3.3 and pET11a. The sequence cloned is shown in Table 20.

4. Generation of host strains for T7 expression

A λDE3 lysogenisation kit was obtained from Novagen Inc and used according to the instructions to generate T7 expression hosts from the *E.coli* strains listed in Table 18. Plasmids pLysS and pLysE and *E.coli* expression hosts BL21 and BL21(DE3) were obtained from Novagen Inc.

Example 1

E.coli strains BL21(DE3), BL21(DE3) pLysS, BL21(DE3) pLysE were transformed separately with plasmids (described below , Table 1) expressing human TNFα. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. Host strains BL21(DE3), BL21(DE3) pLysS and BL21(DE3)pLysE are used extensively in the art and are freely available to the public, for example, from Novagen Inc (Madison ,USA). The genotype of BL21(DE3) is F^-, ompT, $hsdS_B(r_B^- m_B^-)$, gal, dcm, (DE3).

Table 1

Host	Plasmid	Description	Recombinant Designation No
BL21(DE3)	pZen1798	pET11a:TNFα	BL21(DE3) pZen1798
BL21(DE3)	pZen1830	pZT7#2.0:TNFα	BL21(DE3) pZen1830
BL21(DE3)	pZen1832	pZT7#2.1:TNFα	BL21(DE3) pZen1832
BL21(DE3)	pZen1835	pZT7#3.0:TNFα	BL21(DE3) pZen1835
BL21(DE3)	pZen1836	pZT7#3.1:TNFα	BL21(DE3) pZen1836

BL21(DE3)	pZen1826	pZT7#3.2:TNFα	BL21(DE3) pZen1826
BL21(DE3)	pZen1827	pZT7#3.3:TNFα	BL21(DE3) pZen1827
BL21(DE3) pLysS	pZen1798	pET11a:TNFα	BL21(DE3) pLysS/pZen1798
BL21(DE3) pLysE	pZen1798	pET11a:TNFα	BL21(DE3) pLysE/pZen1798
BL21(DE3) pLysS	pZen1830	pZT7#2.0:TNFα	BL21(DE3) pLysS/pZen1830
BL21(DE3) pLysE	pZen1830	pZT7#2.0:TNFα	BL21(DE3) pLysE/pZen1830

An aliquot of each recombinant strain was removed from stock and streaked onto L-agar plates (supplemented with ampicillin (50μg/ml) or tetracycline (10μg/ml) and/or chloramphenicol (1μg/ml) to maintain selection as appropriate) and incubated at 37°C for 16 hours. An aliquot of each culture was then resuspended in 10ml of sterile PBS (phosphate buffered saline solution; 8g/L sodium chloride, 0.2g/L potassium chloride, 0.2g/L potassium di-hydrogen orthophosphate, 1.15g/L magnesium chloride) and used to inoculate to OD_{550} = 0.1 each of two 250ml Erlenmeyer flasks containing 75ml of L-broth (10g/L tryptone (Difco), 5g/L yeast extract (Difco), 5g/L sodium chloride; pH 7.2) supplemented with tetracycline (10μg/ml) or ampicillin (50μg/ml) and/or chloramphenicol (1μg/ml) as appropriate. The flasks were then incubated at 37°C on a reciprocating shaker. Growth was monitored until OD_{550} = 0.4-0.5. At this point cultures were induced by adding the inducer, IPTG (isopropyl-β-D-1-thiogalactopyranoside), to a final concentration of 1mM to one flask from each set (of two) for each recombinant strain. The second flask was not induced. Both flasks for each recombinant strain were incubated under the conditions described above for a further 24h. The accumulation level of hTNFα in the induced cultures was determined by laser densitometry scanning of Coomassie blue stained gels following SDS-PAGE of the sampled bacteria. The basal accumulation level of hTNFα in the un-induced cultures was determined by Western blot analysis (using an anti-hTNFα antibody) following SDS-PAGE of the sampled bacteria. The accumulation level in terms of molecules of hTNFα per cell was then determined by laser densitometry scanning of the blots (prepared using known standards together with the "unknowns") as is well established in the art. The results are summarised below (Table 2).

Table 2

VECTOR	hTNFα Accumulation level: Basal and Induced			
	Operator[1]	Basal % TMP[2]	Basal mol/cell[3]	Induced % TMP[2]
pET11a	**single native lacO**	3.06	98000	30
pZT7#2.0	**single native lacO**	2.66	85000	33
pZT7#3.0	**dual native lacO**	0.14	4500	31
pZT7#2.1	**single ppop lacO**	1.22	39000	33
pZT7#3.1	**dual ppop/native lacO**	0.59	19000	37
pZT7#3.2	**dual native/ppop lacO**	0.08	2400	39
pZT7#3.3	**dual ppop lacO**	0.016	500	44
pET11a/pLysS	**single native lacO**	0.045	1400	2.2
pET11a/pLysE	**single native lacO**	0.025	800	0.21
pZT7#2.0/pLysS	**single native lacO**	0.02	600	1.2
pZT7#2.0/pLysE	**single native lacO**	nd[4]	nd[4]	0.15

(1): described more fully in the text

(2): TMP = Total Microbial Protein

(3): mol/cell = molecules of hTNFα per cell; detection limit: 250 molecules/cell

(4): nd = not detected (Western blot)

The data presented above clearly shows that the level of basal expression of a heterologous protein is still high using the current established art (single native lac operator:

vectors pET11a/ pZT7#2.0). Basal expression can be reduced with pET11a/pZT7#2.0 expression systems by use of host strains co-transformed with plasmids expressing T7 lysozyme (pLysS/pLysE). However, the induced productivity is severely compromised.

Surprisingly, the dual native lac operator sequence works with a T7 promoter driven system reducing basal expression levels significantly whilst not influencing induced productivity.

More surprisingly, the dual perfect palindrome operator performs the best in reducing basal expression levels yet further without compromising induced productivity. This is totally un-expected given that the use of a single perfect palindrome operator with a T7 promoter driven system does not yield a significant improvement in reducing basal expression. Other combinations of the native and ppop lac operators may also be used e.g. pZT7#3.2 and pZT7#3.1.

Example 2

E.coli strains MSD 623(DE3), MSD 624(DE3), MSD 68(DE3), MSD 101(DE3) and MSD 522(DE3) - see Table 18, were transformed separately with plasmids pET11a:TNF, pZT7#2.0:TNF, pZT7#2.1:TNF, pZT7#3.0:TNF, pZT7#3.1:TNF, pZT7#3.2:TNF and pZT7#3.3:TNF expressing human TNFα (described previously (Table 1)). The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. The level of basal and induced expression/accumulation of hTNFα was determined exactly as described in Example 1. The basal (un-induced) and IPTG induced level of hTNFα expression/ accumulation are summarised below in Table 3 and 4 respectively. The data obtained using host strain BL21(DE3), described in Example 1, is included for reference.

Table 3

VECTOR	Host Strain/Basal hTNFα accumulation level: molecules per cell*					
	BL21 (DE3)	MSD623 (DE3)	MSD624 (DE3)	MSD68 (DE3)	MSD101 (DE3)	MSD522 (DE3)
pET11a	98000	7600	4200	2300	4300	6500
pZT7#2.0	85000	7000	2800	3000	5300	3000
pZT7#2.1	39000	5000	2000	1300	3300	2500
pZT7#3.0	4500	650	300	250	400	700
pZT7#3.1	19000	1100	400	300	900	800

pZT7#3.2	2400	1000	300	250	400	350
pZT7#3.3	500	800	400	250	300	800

*Detection limit: 250 molecules/cell.

Table 4

VECTOR	**Host Strain/hTNFα accumulation after induction (%TMP)***					
	BL21 (DE3)	**MSD623 (DE3)**	**MSD624 (DE3)**	**MSD68 (DE3)**	**MSD101 (DE3)**	**MSD522 (DE3)**
pET11a	30	33	29	37	32	34
pZT7#2.0	33	29	33	33	38	16
pZT7#2.1	33	36	46	47	44	47
pZT7#3.0	31	28	39	36	34	36
pZT7#3.1	37	27	38	37	38	36
pZT7#3.2	39	32	48	47	43	42
pZT7#3.3	44	38	48	47	45	39

(*): TMP = Total Microbial Protein

The data presented above show that pZT7#3.0, pZT7#3.1, pZT7#3.2 and pZT7#3.3 decrease the level of basal expression (compared to pET11a/pZT7#2.0) with all host strains tested without adversely influencing the induced productivity. pZT7#3.3 is consistently superior for all host strains tested.

Example 3

Aliquots of *E.coli* strains MSD 101(DE3) pZen1798 (pET11a:TNF) and MSD 101(DE3) pZen1827 (pZT7#3.3:TNF) from glycerol stocks at -80°C were streaked onto plates of L-agar (supplemented with ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate to maintain selection) and incubated at 37°C for 16 hours. An aliquot of each culture was then resuspended in 10ml of sterile PBS and used to inoculate, to OD_{550} = 0.1, 250ml Erlenmeyer flasks containing 75ml of :

1. L-broth (no glucose),
2. L-broth + 1g/L glucose,
3. M9 minimal medium with 2g/L glucose, and
4. M9 minimal medium with 4g/L glycerol.

All the growth media used above were supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate. The composition of M9 minimal medium is given in Table 5 below. The composition of L-broth medium has been described previously.

Table 5: Composition of M9 minimal medium

Component	g/L deionised water*
di-sodium hydrogen orthophosphate	6.0
potassium di-hydrogen orthophosphate	3.0
ammonium chloride	1.0
sodium chloride	0.5
magnesium sulphate hepta-hydrate	1mM
calcium chloride di-hydrate	0.1mM
thiamine	4µg/ml
casein hydrolysate (Oxoid L41)	0.2

(* Final pH adjusted to pH 7.0)

The flasks were then incubated at 37°C on a reciprocating shaker for 24 hours. The basal accumulation level of hTNFα in the un-induced cultures (summarised below in Table 6) was determined exactly as previously described.

Table 6

VECTOR	Growth medium/Basal hTNFα accumulation level (molecules per cell)*			
	L-broth no glucose	L-broth 1g/L glucose	M9 minimal 2g/L glucose	M9 minimal 4g/L glycerol
pET11a	58000	24000	5100	64000
pZT7#3.3	320	250	<250	<250

*Detection limit: 250 molecules/cell

This example demonstrates the wide utility of vector pZT7#3.3. Whereas pET11a shows growth medium dependant repression of expression, vector pZT7#3.3 in sharp contrast shows tight repression in both L-broth and M9 minimal growth media. This was surprising and totally unexpected. L-broth and M9 minimal growth media represent the two basic forms of microbial growth media: complex (L-broth) and minimal salts (M9).

Example 4

The utility of vector pZT7#3.3 for the cloning and overproduction of toxic proteins is exemplified in this example using recombinant bovine pancreatic deoxyribonuclease (DNase 1). Host strains BL21 (non-expressing host background) and BL21(DE3) (expressing background) were transformed as follows. Competant cells prepared using the $CaCl_2$ method (Sambrook, Fritsch and Maniatis, 1989, "Molecular Cloning", 2nd Edition, Cold Spring Harbour Press, New York) were transformed with a range of plasmid DNA concentrations using the "heat shock" method (Sambrook, Fritsch and Maniatis, 1989, "Molecular Cloning", 2nd Edition, Cold Spring Harbour Press, New York) with pZen2006 (pAD10, the pET11 derivative expressing DNase 1 described previously), pZen1980 (pZT7#3.3:DNase 1) and pZen1827 (pZT7#3.3:TNF) expressing hTNFα (used as a control: relatively non-toxic gene product). The transformation efficiency of each host-plasmid combination was determined as is well described in the art. The results are summarised below in Table 7.

Table 7

VECTOR	**Host/Transformation Efficiency: transformants/μg plasmid DNA***	
	BL21: non expressing background	**BL21(DE3): expressing background**
pET11a	no clones	
pAD10 Dnase 1	2.2×10^5 ($+/- 2.2 \times 10^4$)	120 (+/- 170)
pZT7#3.3	2.7×10^5	3.7×10^5

Dnase 1	(+/- 3.6 x 10^4)	(+/- 1.4 x 10^4)
pZT7#3.3	2.5 x 10^5	3.5 x 10^5
hTNFα	(+/- 3.7 x 10^4)	(+/- 6.4 x 10^4)

*Data from three separate experiments (n=3)

The above data clearly exemplifies the tight control of basal expression that is achieved using pZT7#3.3. The results show that pZT7#3.3:DNase 1 is sufficiently repressed to support transfer into a cell expressing T7 RNA polymerase (BL21(DE3)) without a deleterious effect on cell viability. Transformation efficiencies achieved are equivalent to those obtained with transformation into BL21 (no T7 RNA polymerase) or to those with a relatively non-toxic gene product pZT7#3.3:hTNFα. If expression of DNase 1 had been leaky the cells would have been killed. This is in contrast to the results obtained with pET11a and pAD10. It will be clearly evident to those experienced in the art how pZT7#3.3 may be used to circumvent the problem of the deleterious effect that a heterologous protein can have on growth and productivity of recombinant cells.

The expression/accumulation of DNase 1 using BL21(DE3) pZen1980 (pZT7#3.3:DNase 1) was determined by taking single colony of a BL21(DE3) pZen1980 transformant from the experiment described above and using this to inoculate a single Erlenmeyer flask containing 75ml of L-broth (1g/L glucose, 10μg/ml tetracycline). The flask was incubated at 37°C on a reciprocating shaker for 16 hours. This culture was then used to inoculate fresh L-broth (1g/L glucose, 10μg/ml tetracycline) to $OD_{550} = 0.1$. The flask was then incubated at 37°C on a reciprocating shaker until the growth reached $OD_{550} = 0.5$. The culture was then induced by adding IPTG (0.5mM final) and the incubation continued, under the conditions described, for a further 4 hours. The cells were harvested and the cell pellet stored at -20°C. The cell pellet was thawed and resuspended (10% w/v (wet weight)) in lysis buffer (10mM Tris; pH 7.6, 2mM calcium chloride, 100μM benzamidine and 100μM phenylmethylsulfonyl flouride (PMSF)). The cell suspension was then sonicated (20-30 second bursts followed by a period on ice) until examination of the suspension by light microscopy indicated >95% cell breakage. The cell debris was removed by centrifugation (4°C, 25000 x *g*, 20 minutes) and DNase 1 activity in the supernatant determined by adding 100μl of the cleared supernatant to 1ml of Kunitz assay buffer (10mM Tris; pH 8.0, 0.1mM calcium chloride, 1mM magnesium chloride and 50μg calf thymus DNA). One "Kunitz Unit" is that

amount of DNase 1 that causes an increase in the A_{260nm} of 0.001/min. The results are summarised below in Table 8.

Table 8

Host/Vector	Growth OD_{550} at harvest	Dnase 1 Activity: Kunitz Units per litre of culture
BL21(DE3) pZen1980 (pZT7#3.3:DNase 1)	4.0	2×10^5

Doherty *et al* (Gene, 1993, 136, pp337-340) found that on transformation of BL21(DE3) with pAD10, no viable bacterial colonies were obtained. This is essentially similar (given the standard deviation in the data) to our observations (described above in Table 7). Even with BL21(DE3) pLysS, Doherty *et al* found that transformants had poor viability when transferred to liquid media. In sharp contrast BL21(DE3) transformed with pZen1980 (pZT7#3.3:DNase 1) achieves high transformation efficiencies which are equivalent to those achieved using a non-expressing host background and moreover BL21(DE3) pZen1980 transformants demonstrate high viability in liquid culture and retain the ability to express biologically active DNase 1 even after sub-culture.

Example 5

Aliquots of *E.coli* strains MSD 101(DE3) pZen1798 (pET11a:TNF) and MSD 101(DE3) pZen1827 (pZT7#3.3:TNF) from glycerol stocks at -80°C were streaked onto plates of L-agar (supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate to maintain selection) and incubated at 37°C for 16 hours. An aliquot of each culture was used to inoculate each of two 250ml Erlenmeyer flasks containing 75ml of M9 minimal medium (2g/L glucose supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate). The cultures were incubated at 37°C for 16 hours on a reciprocal shaker and used to inoculate separately to $OD_{550} = 0.1$ each of five 2L Erlenmeyer flasks containing 600ml of M9 minimal medium (2g/L glucose supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate). The composition of M9 minimal medium is given in Table 5. The flasks were incubated at 37°C on a reciprocal shaker and the growth monitored periodically by measuring the OD_{550} of the culture. When the growth reached $OD_{550} = 0.5$ the cultures were induced by adding the inducer IPTG to

each flask (0.25mM, 0.075mM, 0.05mM, 0,025mM and 0.01mM (final)). The incubation was continued under the conditions described for a further 10 hours during which samples were taken for measurement of growth, accumulation of hTNFα and total microbial protein within the bacterial cells. The accumulation level of hTNFα was measured by scanning Coomassie blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria as is well known in the art. The level of total microbial protein was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) used in accordance with the manufacturers instructions. The accumulation level of biomass was determined by calculating the dry weight of the biomass from the OD_{550} measurements as is well established in the art. The specific productivity (Q_p) of hTNFα (mg hTNFα produced per gram dry weight cells per hour) was calculated for each sample point during the induction period using protocols well established in the art. The $Q_{p(max)}$ (peak specific productivity) and maximum hTNFα accumulation level (% total microbial protein) attained with each IPTG concentration used for induction) are summarised in Figures 13 and 14 respectively (*TMP = Total microbial protein).

These results show that adding IPTG to the medium at increasing concentrations induces expression in a dose-dependant manner with pZT7#3.3. However, with pET11a expression is induced to near maximum levels even at very low concentrations of inducer. It will be evident how this surprising and unexpected property of pZT7#3.3 allows those skilled in the art to control production of heterologous proteins over a wide range of expression levels so that an optimum level of expression can be identified. This is exemplified by Examples 9 and 10.

Example 6

E.coli strains BL21(DE3) and BL21(DE3) pLysS were transformed separately with plasmid pZen1911 (pET11a:LARd1(1275-1613) expressing LARd1(1275-1613). *E.coli* strains MSD460(DE3) and MSD460(DE3) pLysS were transformed separately with plasmid pZen1914 (pET11a:ZAP70(4-260)-6His) expressing ZAP70(4-260)-6His. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. An aliquot of each culture was removed from stock and streaked separately on to L-agar plates (supplemented with ampicillin (50μg/ml) or ampicillin (50μg/ml) and chloramphenicol (1μg/ml) as appropriate to maintain selection) to separate single colonies after growth for 16 hours at 37°C . A single colony of each culture was then inoculated separately into a 250ml Erlenmeyer flasks containing 75ml of L-broth (+ 1g/L glucose and ampicillin (50μg/ml) or ampicillin (50μg/ml) and chloramphenicol (1μg/ml)) as

appropriate). The flasks were incubated at 37°C on a reciprocating shaker for 16 hours. Each of these seeder cultures was then used to inoculate separately 250ml Erlenmeyer flasks containing 75ml L-broth (1g/L glucose and ampicillin (50µg/ml) or ampicillin (50µg/ml) and chloramphenicol (1µg/ml)) as appropriate) to $OD_{550} = 0.1$. The flasks were then incubated at 20°C on a reciprocating shaker until the growth reached $OD_{550} = 0.5$. The cultures were then induced by adding IPTG (0.5mM for LARd1(1275-1613) and 2mM for ZAP70(4-260)-6His) and the incubation continued, under the conditions described, for a further 24 hours. LARd1(1275-1613) and ZAP70(4-260)-6His accumulation was measured in the seeder cultures and IPTG induced cultures described above by scanning Coomassie blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria as is well known in the art. The results are summarised below in Table 9.

Table 9

HOST STRAIN	PLASMID	Accumulation level %TMP*	
		BASAL: (seeder culture)	INDUCED
LARd1(1275-1613)			
BL21(DE3)	pZen1911	18	40
BL21(DE3) pLysS	pZen1911	nd(1)	13
ZAP70(4-260)-6His			
MSD460(DE3)	pZen1914	0.4	12
MSD460(DE3) pLysS	pZen1914	nd(1)	nd(1)

*TMP: Total microbial protein

1. not detected on Coomassie blue stained SDS-PAGE gels

This example further exemplifies the poor performance of pET11a in terms of high basal expression. Reducing basal expression in the absence of inducer using pLysS reduces the level of basal expression but adversely influences induced productivity.

Example 7

E.coli strain BL21(DE3) was transformed separately with plasmids pZen1977 (pET11a:MCP-1(9-76)) and pZen1848 (pZT7#3.3:MCP-1(9-76)) expressing MCP-1(9-76). The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. An aliquot of each culture was removed from stock and streaked separately on to L-agar plates

(supplemented with ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate to maintain selection) to separate single colonies after growth for 16 hours at 37°C . A single colony of each culture was then inoculated into each of two 250ml Erlenmeyer flasks containing 75ml of L-broth (+ 1g/L glucose and ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate). The flasks were incubated at 37°C on a reciprocating shaker for 16 hours. Each of these cultures was then used to inoculate three 250ml Erlenmeyer flasks containing 75ml L-broth (1g/L glucose and ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate) to OD_{550} = 0.1. The flasks were then incubated at 37°C, 30°C and 20°C on a reciprocating shaker until the growth reached OD_{550} = 0.5. The cultures were then induced by adding IPTG (0.25mM final) and the incubation continued, under the conditions described, for a further 5- 24 hours. MCP-1(9-76) accumulation was measured by scanning Coomassie blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria as is well known in the art. Partitioning (solubility) of MCP-1(9-76) in the cytoplasmic (soluble) and pellet (insoluble) fractions of cells was determined by subjecting sampled bacteria to sonication lysis as is well known in the art. The results are summarised below in Table 10.

Table 10

VECTOR	Temperature °C	Induction time (h)	Accumulation MCP-1(9-76) %TMP*	Solubility %
pET11a: MCP-1(9-76)	20	24	7	100
pET11a: MCP-1(9-76)	30	24	3	100
pET11a: MCP-1(9-76)	37	5	4	100
pZT7#3.3: MCP-1(9-76)	20	24	12	100
pZT7#3.3: MCP-1(9-76)	30	24	14	95
pZT7#3.3: MCP-1(9-76)	37	5	22	80

*TMP = Total microbial protein

The utility of vector pZT7#3.3 for high level soluble accumulation of MCP-1(9-76) is clearly evident from the data presented above.

Example 8

E.coli strain MSD460(DE3) was transformed separately with plasmids pZen1914 (pET11a:ZAP70(4-260)-6His) and pZen1913 (pZT7#3.3:ZAP70(4-260)-6His) expressing ZAP70(4-260)-6His. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. An aliquot of each culture was removed from stock and streaked separately on to L-agar plates (supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate to maintain selection) to separate single colonies after growth for 16 hours at 37°C. A single colony of each culture was then inoculated into each of two 250ml Erlenmeyer flasks containing 75ml of L-broth (+ 1g/L glucose and ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate). The flasks were incubated at 30°C on a reciprocating shaker for 16 hours. Each of these cultures was then used to inoculate a 250ml Erlenmeyer flask containing 75ml L-broth (1g/L glucose and ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate) to $OD_{550} = 0.1$. The flasks were then incubated at 20°C on a reciprocating shaker until the growth reached $OD_{550} = 0.5$. The cultures were then induced by adding IPTG (2mM (final)) and the incubation continued, under the conditions described, for a further 24 hours.

ZAP70(4-260)-6His accumulation after induction was measured by scanning Coomassie blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria as is well known in the art. Partitioning (solubility) of ZAP70(4-260)-6His in the cytoplasmic (soluble) and pellet (insoluble) fractions of cells was determined by subjecting sampled bacteria to sonication lysis as is well known in the art. The sonication lysis buffer included protease inhibitors (1mM phenylmethylsulphonyl flouride (PMSF),1mM benzamidine and 1mM iodoacetamide) to reduce proteolytic degradation of ZAP70(4-260)-6His during sample processing. The results are summarised below in Table 11.

Table 11

VECTOR	ZAP70(4-260)-6His Accumulation %TMP*	Solubility %
pET11a: ZAP70(4-260)-6His	11	<5
pZT7#3.3: ZAP70(4-260)-6His	11	70

*TMP = Total microbial protein

The utility of vector pZT7#3.3 for the soluble accumulation of ZAP70(4-260)-6His is clearly exemplified by the data in the table above.

Example 9

E.coli strain MSD624(DE3) was transformed separately with plasmids pZen1953 (pET11a:CPB [D253K]-6His-cmyc) and pZen1954 (pZT7#3.3:CPB[D253K]-6His-cmyc) expressing CPB[D253K]-6His-cmyc. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. An aliquot of each culture was removed from stock and streaked separately on to L-agar plates (supplemented with ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate to maintain selection) to separate single colonies after growth for 16 hours at 37°C. A single colony of each culture was then inoculated into each of two 250ml Erlenmeyer flasks containing 75ml of L-broth (+ 1g/L glucose and ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate). The flasks were incubated at 37°C on a reciprocating shaker for 16 hours. Each of these cultures was then used to inoculate ten 2L Erlenmeyer flasks containing 600ml L-broth (1g/L glucose and ampicillin (50µg/ml) or tetracycline (10µg/ml) as appropriate) to $OD_{550} = 0.1$. The flasks were then incubated at 20°C on a reciprocating shaker until the growth reached $OD_{550} = 0.5$. The cultures were then induced by adding IPTG (0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.075, 0.1 and 0.25mM IPTG (final)) and the incubation continued, under the conditions described, for a further 48 hours. The cells were then harvested (4°C, 25000 x *g*, 20 minutes) and subjected to osmotic shock cell fractionation (as is well known in the art) to isolate the cellular fraction containing proteins partitioned in the soluble *E.coli* periplasmic fraction. The accumulation of biologically active CPB[D253K]-6His-cmyc in the soluble *E.coli* periplasmic extract was determined by measuring the release of hippuric acid from the substrate hippuryl-L-glutamine as follows.

Cell free periplasmic extract (125µl) was added to a test tube containing 100µl 25mM Tris buffer (pH 7.5), 2.5µl 100mM zinc chloride and 0.5mM substrate (hippuryl-L-glutamine). This was incubated at 37°C for 24 hours. The reaction was stopped by adding 250µl "Stop solution" (40% methanol (HPLC grade), 60% 50mM phosphate buffer (Sigma P8165), 0.2% w/v trichloroacetic acid. After mixing any precipitate formed was removed by centrifugation (4°C, 16000 x *g*, 3 minutes). The amount of hippuric acid in the cleared supernatant was then determined using HPLC as is well established in the art.

The accumulation of biologically active CPB[D253K]-6His-cmyc was determined by reference to a standard curve prepared with purified active recombinant CPB[D253K]-6His-cmyc and hippuric acid (Sigma H6375).

The accumulation in the periplasm of *E.coli* of biologically active CPB[D253K]-6His-cmyc (as μg active material/L of culture) is presented in Figure 15.

Example 10

E.coli strain MSD624(DE3) was transformed separately with plasmids pZen1999 (pET11a:A5B7(Fab')$_2$) and pZen1997 (pZT7#3.3: A5B7(Fab'$_2$) expressing A5B7(Fab')$_2$. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C. An aliquot of each culture was removed from stock and streaked separately on to L-agar plates (supplemented with ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate to maintain selection) to separate single colonies after growth for 16 hours at 37°C. A single colony of each culture was then inoculated into each of two 250ml Erlenmeyer flasks containing 75ml of L-broth (+ 1g/L glucose and ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate). The flasks were incubated at 37°C on a reciprocating shaker for 16 hours. Each of these cultures was then used to inoculate thirteen 2L Erlenmeyer flasks containing 600ml L-broth (1g/L glucose and ampicillin (50μg/ml) or tetracycline (10μg/ml) as appropriate) to $OD_{550} = 0.1$. The flasks were then incubated at 20°C on a reciprocating shaker until the growth reached $OD_{550} = 0.5$. The cultures were then induced by adding IPTG (0.005, 0.01, 0.025, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.2 and 0.25mM IPTG (final)) and the incubation continued, under the conditions described, for a further 48 hours. The cells were then harvested (4°C, 25000 x *g*, 20 minutes) and subjected to osmotic shock cell fractionation (as is well known in the art) to isolate the cellular fraction containing proteins partitioned in the soluble *E.coli* periplasmic fraction. The accumulation of biologically active A5B7(Fab'$_2$/A5B7(Fab') in the soluble *E.coli* periplasmic extract was estimated by determining the binding of A5B7(Fab'$_2$/A5B7(Fab') to human tumour carcinoembryonic antigen (CEA) in an ELISA assay. The accumulation in the periplasm of *E.coli* of biologically active A5B7(Fab'$_2$/A5B7(Fab') (as mg active material/L of culture) is presented in Figure 16.

With both proteins (described in Examples 9 and 10 above), pZT7#3.3 accumulates higher levels of active product in the periplasm of *E.coli* than pET11a. The data presented in Figures 15-16 clearly demonstrates how the modulation characteristics of pZT7#3.3 can be exploited to

optimise recombinant protein yields. These examples exemplify the use of pZT7#3.3 vector for secretion. However, it will be readily apparent to those skilled in the art how the basal level of expression/modulation of expression characteristics of pZT7#3.3 also facilitates the expression and accumulation of heterologous membrane proteins.

Table 12

PCR primer #1 (lac I 5′-3′)

GATGCTATAATGCATGACACCATCGAATGGCGCAA

PCR primer #2 (lacI 3′-5′)

CAGTATGCACAGTATGCATTTACATTAATTGCGTTGCGCTC

5′-3′ oligomer #3

AATTCcagaCATATGGTACCAGTACTctatACTAGTtgaaGGATCCatgcCTCGAGaacgCTGCA
GagctAAGCTTgacaAGATCTaa

3′-5′ oligomer #4

gatcttAGATCTtgtcAAGCTTagctCTGCAGcgttCTCGAGgcatGGATCCttcaACTAGT
atagAGTACTGGTACCATATGtctgG

5′-3′ oligomer #5

agcttAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTA
GCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGa

3′-5′ oligomer #6

gatctCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTG
CTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTa

5′-3′ oligomer #7

tcgagGCATTGTCCTCTTAGTTAAATGGATATAACGAGCCCCTCCTAAGGGCTAATTGCA
GGTTCGATTCCTGCAGGGGACTCCActgca

3′-5′ oligomer #8

gTGGAGTCCCCTGCAGGAATCGAACCTGCAATTAGCCCTTAGGAGGGGCTCGTTATAT
CCATTTAACTAAGAGGACAATGCc

5′-3′ oligomer #9

cctATTATATTACTAATTAATTGGGGACCCTAGAGGTCCCCTTTTTTATTTTAAAAccatgg
aaccaaccg

3′-5′ oligomer #10

aattcggttggttccatggTTTTAAAATAAAAAAGGGGACCTCTAGGGTCCCCAATTAATTAGTA
ATATAATagg

5′-3′ oligomer #11

aattcCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCT
AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAca

3′-5′ oligomer #12

tatgTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGC
TCACAATTCCCCTATAGTGAGTCGTATTAATTTCGg

5′-3′ oligomer #13

aattcCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGCTCACAATTCCCCTCTA
GAAATAATTTTGTTTAACTTTAAGAAGGAGATATAca

3′-5′ oligomer #14

tatgTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTGAGCGCT
CACAATTCCCCTATAGTGAGTCGTATTAATTTCGg

5′-3′ oligomer #15

catggACTGGTTAACAACCAACCGGAATTGTGAGCGGATAACAATTCCTCCAAGAACAA
CCATCCTAGCAACACGGCGGTCCCCg

3′-5′ oligomer #16

aattcGGGGACCGCCGTGTTGCTAGGATGGTTGTTCTTGGAGGAATTGTTATCCGCTCAC
AATTCCGGTTGGTTGTTAACACGTc

5′-3′ oligomer #17

catggACGTGTTAACAACCAACCGGAATTGTGAGCGCTCACAATTCCATCCAAGAACAA
CCATCCTAGCAACACGGCGGTCCCCg

3′-5′ oligomer #18

aattcGGGGACCGCCGTGTTGCTAGGATGGTTGTTCTTGGATGGAATTGTGAGCGCTCAC
AATTCCGGTTGGTTGTTAACACGTC

TABLE 13

hTNFα sequence

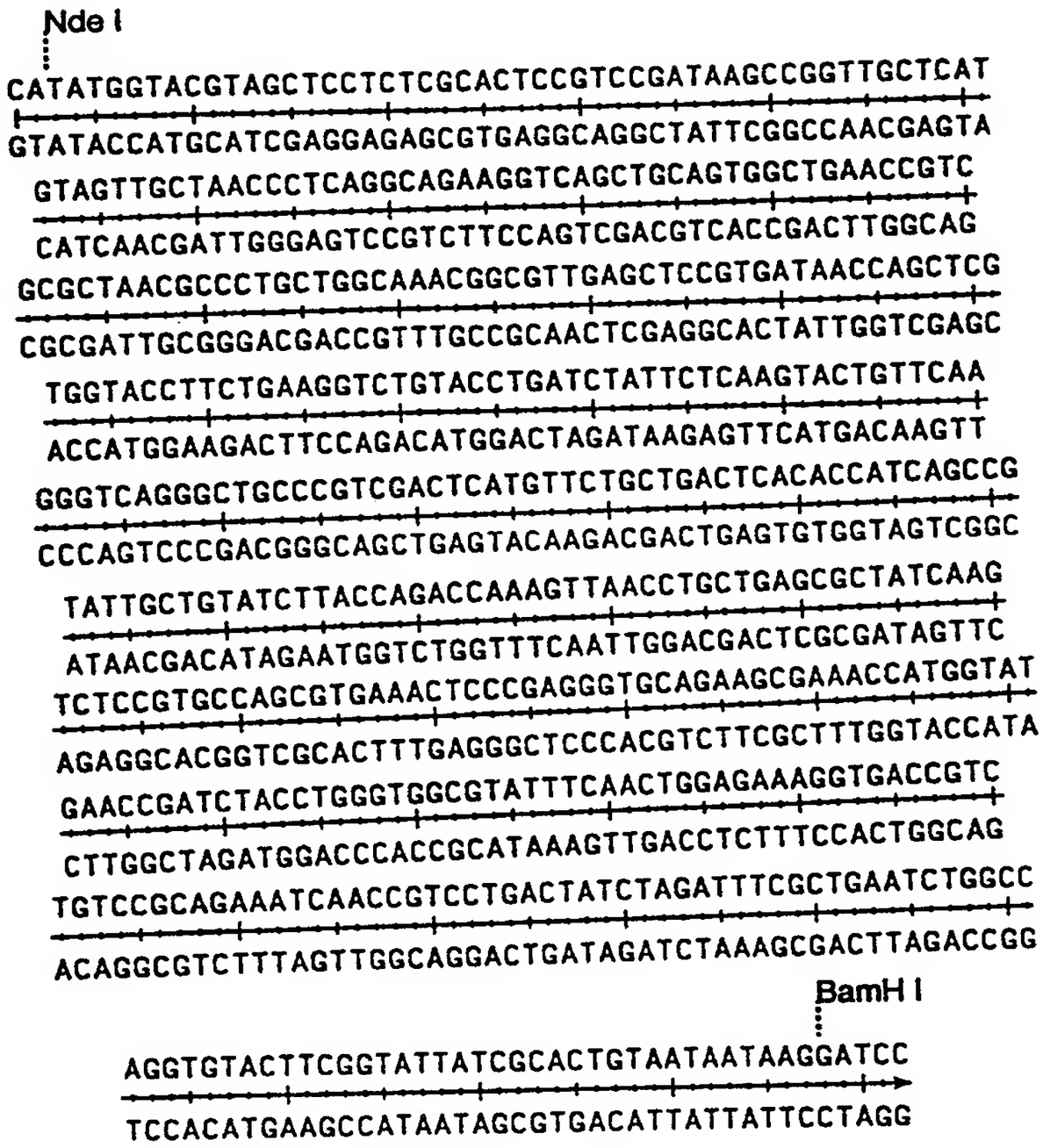

Table 14

ZAP70 (4-260) 6HIS sequence

Nde I

CATATGCCCGCGGCGCACCTGCCCTTCTTCTACGGCAGCATCTCGCGTGCCGAGGCCGAGGAGCACCTGAAGCTGGCGGG
GTATACGGGCGCCGCGTGGACGGGAAGAAGATGCCGTCGTAGAGCGCACGGCTCCGGCTCCTCGTGGACTTCGACCGCCC 80

CATGGCGGACGGGCTCTTCCTGCTGCGCCAGTGCCTGCGCTCGCTGGGCGGCTATGTGCTGTCGCTCGTGCACGATGTGC
GTACCGCCTGCCCGAGAAGGACGACGCGGTCACGGACGCGAGCGACCCGCCGATACACGACAGCGAGCACGTGCTACACG 16

GCTTCCACCACTTTCCCATCGAGCGCCAGCTCAACGGCACCTACGCCATTGCCGGCGGCAAAGCGCACTGTGGACCGGCA
CGAAGGTGGTGAAAGGGTAGCTCGCGGTCGAGTTGCCGTGGATGCGGTAACGGCCGCCGTTTCGCGTGACACCTGGCCGT 24

GAGCTCTGCGAGTTCTACTCGCGCGACCCCGACGGGCTGCCCTGCAACCTGCGCAAGCCGTGCAACCGGCCGTCGGGCCT
CTCGAGACGCTCAAGATGAGCGCGCTGGGGCTGCCCGACGGGACGTTGGACGCGTTCGGCACGTTGGCCGGCAGCCCGGA 32

CGAGCCGCAGCCGGGGGTCTTCGACTGCCTGCGAGACGCCATGGTGCGTGACTACGTGCGCCAGACGTGGAAGCTGGAGG
GCTCGGCGTCGGCCCCCAGAAGCTGACGGACGCTCTGCGGTACCACGCACTGATGCACGCGGTCTGCACCTTCGACCTCC 40

GCGAGGCCCTGGAGCAGGCCATCATCAGCCAGGCCCCGCAGGTGGAGAAGCTCATTGCTACGACGGCCCACGAGCGGATG
CGCTCCGGGACCTCGTCCGGTAGTAGTCGGTCCGGGGCGTCCACCTCTTCGAGTAACGATGCTGCCGGGTGCTCGCCTAC 48

CCCTGGTACCACAGCAGCCTGACGCGTGAGGAGGCCGAGCGCAAACTTTACTCTGGGGCGCAGACCGACGGCAAGTTCCT
GGGACCATGGTGTCGTCGGACTGCGCACTCCTCCGGCTCGCGTTTGAAATGAGACCCCGCGTCTGGCTGCCGTTCAAGGA 56

GCTGAGGCCGCGGAAGGAGCAGGGCACATACGCCCTGTCCCTCATCTATGGGAAGACGGTGTACCACTACCTCATCAGCC
CGACTCCGGCGCCTTCCTCGTCCCGTGTATGCGGGACAGGGAGTAGATACCCTTCTGCCACATGGTGATGGAGTAGTCGG 64

AAGACAAGGCGGGCAAGTACTGCATTCCCGAGGGCACCAAGTTTGACACGCTCTGGCAGCTGGTGGAGTATCTGAAGCTG
TTCTGTTCCGCCCGTTCATGACGTAAGGGCTCCCGTGGTTCAAACTGTGCGAGACCGTCGACCACCTCATAGACTTCGAC 72

AAGGCGGACGGGCTCATCTACTGCCTGAAGGAGGCCTGCCCCAACAGCAGTGCCAGCCATCACCATCACCATCACTAATA
TTCCGCCTGCCCGAGTAGATGACGGACTTCCTCCGGACGGGGTTGTCGTCACGGTCGGTAGTGGTAGTGGTAGTGATTAT 80

Bgl II

AAGATCT
807
TTCTAGA

Table 15

LARd1 (aa1275-1623) sequence

Nde I

```
CATATGGTACCAACCCACTCTCCGTCCTCTAAGGATGAGCAGTCGATCGGACTGAAGGACTCCTTGCTGGCCCACTCCTCTGACCCTGTG
GTATACCATGGTTGGGTGAGAGGCAGGAGATTCCTACTCGTCAGCTAGCCTGACTTCCTGAGGAACGACCGGGTGAGGAGACTGGGACAC  90

GAGATGCGGAGGCTCAACTACCAGACCCCAGGTATGCGAGACCACCCACCCATCCCCATCACCGACCTGGCGGACAACATCGAGCGCCTC
CTCTACGCCTCCGAGTTGATGGTCTGGGGTCCATACGCTCTGGTGGGTGGGTAGGGGTAGTGGCTGGACCGCCTGTTGTAGCTCGCGGAG  18

AAAGCCAACGATGGCCTCAAGTTCTCCCAGGAGTATGAGTCCATCGACCCTGGACAGCAGTTCACGTGGGAGAATTCAAACCTGGAGGTG
TTTCGGTTGCTACCGGAGTTCAAGAGGGTCCTCATACTCAGGTAGCTGGGACCTGTCGTCAAGTGCACCCTCTTAAGTTTGGACCTCCAC  27

AACAAGCCCAAGAACCGCTATGCGAATGTCATCGCCTACGACCACTCTCGAGTCATCCTTACCTCTATCGATGGCGTCCCCGGGAGTGAC
TTGTTCGGGTTCTTGGCGATACGCTTACAGTAGCGGATGCTGGTGAGAGCTCAGTAGGAATGGAGATAGCTACCGCAGGGGCCCTCACTG  36

TACATCAATGCCAACTACATCGATGGCTACCGCAAGCAGAATGCCTACATCGCCACGCAGGGCCCCCTGCCCGAGACCATGGGCGATTTC
ATGTAGTTACGGTTGATGTAGCTACCGATGGCGTTCGTCTTACGGATGTAGCGGTGCGTCCCGGGGGACGGGCTCTGGTACCCGCTAAAG  45

TGGAGAATGGTGTGGGAACAGCGCACGGCCACTGTGGTCATGATGACACGGCTGGAGGAGAAGTCCCGGGTAAAATGTGATCAGTACTGG
ACCTCTTACCACACCCTTGTCGCGTGCCGGTGACACCAGTACTACTGTGCCGACCTCCTCTTCAGGGCCCATTTTACACTAGTCATGACC  54

CCAGCCCGTGGCACCGAGACCTGTGGCCTTATTCAGGTGACCCTGTTGGACACAGTGGAGCTGGCCACATACACTGTGCGCACCTTCGCA
GGTCGGGCACCGTGGCTCTGGACACCGGAATAAGTCCACTGGGACAACCTGTGTCACCTCGACCGGTGTATGTGACACGCGTGGAAGCGT  63

CTCCACAAGAGTGGCTCCAGTGAGAAGCGTGAGCTGCGTCAGTTTCAGTTCATGGCCTGGCCAGACCATGGAGTTCCTGAGTACCCAACT
GAGGTGTTCTCACCGAGGTCACTCTTCGCACTCGACGCAGTCAAAGTCAAGTACCGGACCGGTCTGGTACCTCAAGGACTCATGGGTTGA  72

CCCATCCTGGCCTTCCTACGACGGGTCAAGGCCTGCAACCCCCTAGACGCAGGGCCCATGGTGGTGCACTGCAGCGCGGGCGTGGGCCGC
GGGTAGGACCGGAAGGATGCTGCCCAGTTCCGGACGTTGGGGGATCTGCGTCCCGGGTACCACCACGTGACGTCGCGCCCGCACCCGGCG  81

ACCGGCTGCTTCATCGTGATTGATGCCATGTTGGAGCGGATGAAGCACGAGAAGACGGTGGACATCTATGGCCACGTGACCTGCATGCGA
TGGCCGACGAAGTAGCACTAACTACGGTACAACCTCGCCTACTTCGTGCTCTTCTGCCACCTGTAGATACCGGTGCACTGGACGTACGCT  90

TCACAGAGGAACTACATGGTGCAGACGGAGGACCAGTACGTGTTCATCCATGAGGCGCTGCTGGAGGCTGCCACGTGCGGCCACACAGAG
AGTGTCTCCTTGATGTACCACGTCTGCCTCCTGGTCATGCACAAGTAGGTACTCCGCGACGACCTCCGACGGTGCACGCCGGTGTGTCTC  99

                                                    Bgl II
GTGCCTGCCCGCAACCTGTATGCCCACTAATGAAGATCT
CACGGACGGGCGTTGGACATACGGGTGATTACTTCTAGA  1029
```

TABLE 16

Bovine pancreatic DNase 1 sequence

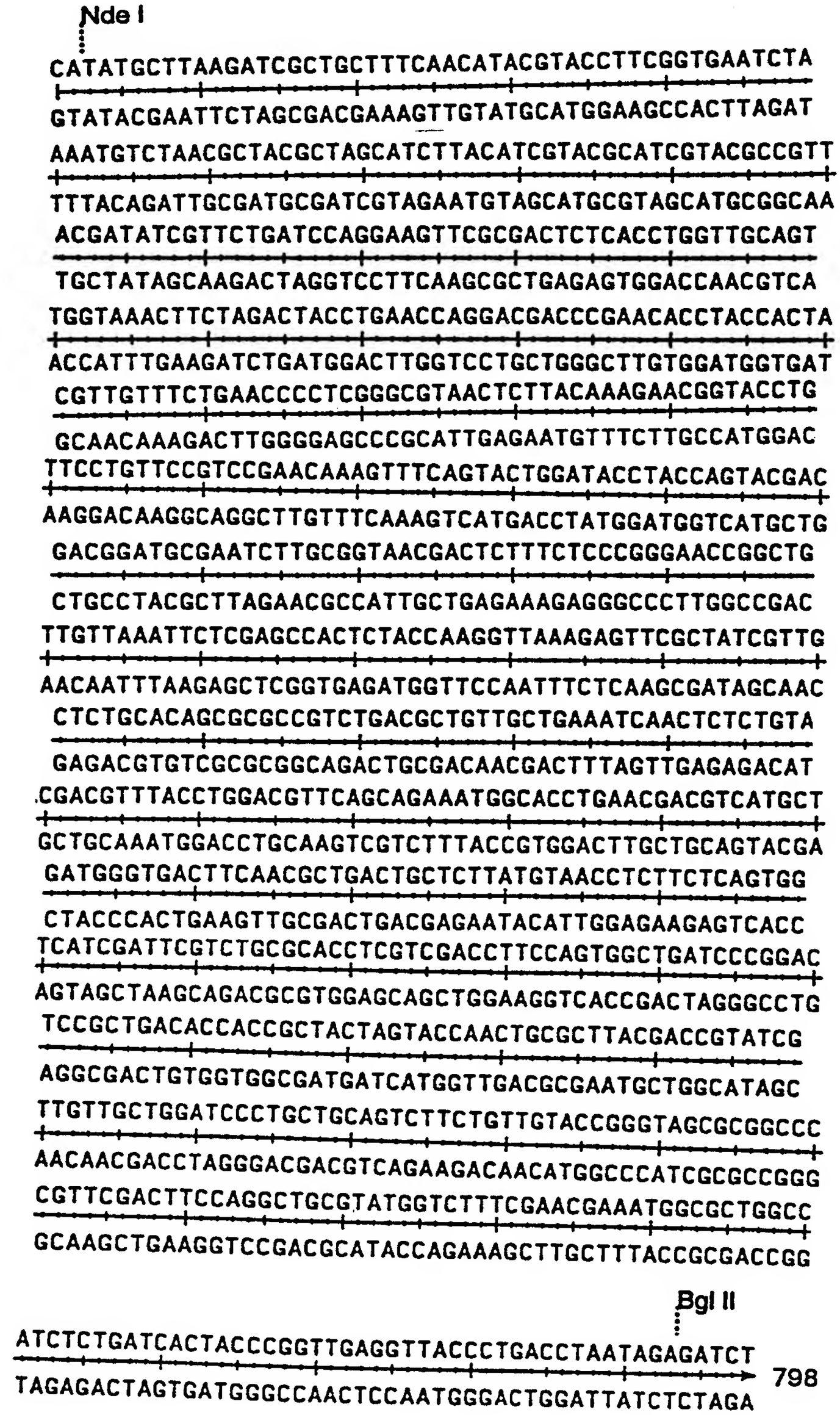

Table 17

human carboxypeptidase B (mutant D253>K) 6His cmyc sequence

```
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                                                                                            90
TACTTTATGGATAACGGATGCCGTCGGCGACCTAACAATAATGAGCGACGGGTTGGTCGGTACCGCCGTTGACCAGTGAGAATGCTCTTC

TACAACAAGTGGGAAACGATAGAGGCTTGGACTCAACAAGTCGCCACTGAGAATCCAGCCCTCATCTCTCGCAGTGTTATCGGAACCACA
                                                                                            18
ATGTTGTTCACCCTTTGCTATCTCCGAACCTGAGTTGTTCAGCGGTGACTCTTAGGTCGGGAGTAGAGAGCGTCACAATAGCCTTGGTGT

TTTGAGGGACGCGCTATTTACCTCCTGAAGGTTGGCAAAGCTGGACAAAATAAGCCTGCCATTTTCATGGACTGTGGTTTCCATGCCAGA
                                                                                            27
AAACTCCCTGCGCGATAAATGGAGGACTTCCAACCGTTTCGACCTGTTTTATTCGGACGGTAAAAGTACCTGACACCAAAGGTACGGTCT

GAGTGGATTTCTCCTGCATTCTGCCAGTGGTTTGTAAGAGAGGCTGTTCGTACCTATGGACGTGAGATCCAAGTGACAGAGCTTCTCGAC
                                                                                            36
CTCACCTAAAGAGGACGTAAGACGGTCACCAAACATTCTCTCCGACAAGCATGGATACCTGCACTCTAGGTTCACTGTCTCGAAGAGCTG

AAGTTAGACTTTTATGTCCTGCCTGTGCTCAATATTGATGGCTACATCTACACCTGGACCAAGAGCCGATTTTGGAGAAAGACTCGCTCC
                                                                                            45
TTCAATCTGAAAATACAGGACGGACACGAGTTATAACTACCGATGTAGATGTGGACCTGGTTCTCGGCTAAAACCTCTTTCTGAGCGAGG

ACCCATACTGGATCTAGCTGCATTGGCACAGACCCCAACAGAAATTTTGATGCTGGTTGGTGTGAAATTGGAGCCTCTCGAAACCCCTGT
                                                                                            54
TGGGTATGACCTAGATCGACGTAACCGTGTCTGGGGTTGTCTTTAAAACTACGACCAACCACACTTTAACCTCGGAGAGCTTTGGGGACA

GATGAAACTTACTGTGGACCTGCCGCAGAGTCTGAAAAGGAGACCAAGGCCCTGGCTGATTTCATCCGCAACAAACTCTCTTCCATCAAG
                                                                                            63
CTACTTTGAATGACACCTGGACGGCGTCTCAGACTTTTCCTCTGGTTCCGGGACCGACTAAAGTAGGCGTTGTTTGAGAGAAGGTAGTTC

GCATATCTGACAATCCACTCGTACTCCCAAATGATGATCTACCCTTACTCATATGCTTACAAACTCGGTGAGAACAATGCTGAGTTGAAT
                                                                                            72
CGTATAGACTGTTAGGTGAGCATGAGGGTTTACTACTAGATGGGAATGAGTATACGAATGTTTGAGCCACTCTTGTTACGACTCAACTTA

GCCCTGGCTAAAGCTACTGTGAAAGAACTTGCCTCACTGCACGGCACCAAGTACACATATGGCCCGGGAGCTACAACAATCTATCCTGCT
                                                                                            81
CGGGACCGATTTCGATGACACTTTCTTGAACGGAGTGACGTGCCGTGGTTCATGTGTATACCGGGCCCTCGATGTTGTTAGATAGGACGA

GCTGGGGGCTCTAAAGACTGGGCTTATGACCAAGGAATCAGATATTCCTTCACCTTTGAACTTCGAGATACAGGCAGATATGGCTTTCTC
                                                                                            90
CGACCCCCGAGATTTCTGACCCGAATACTGGTTCCTTAGTCTATAAGGAAGTGGAAACTTGAAGCTCTATGTCCGTCTATACCGAAAGAG

CTTCCAGAATCCCAGATCCGGGCTACCTGCGAGGAGACCTTCCTGGCAATCAAGTATGTTGCCAGCTACGTCCTGGAACACCTGTACCAC
                                                                                            99
GAAGGTCTTAGGGTCTAGGCCCGATGGACGCTCCTCTGGAAGGACCGTTAGTTCATACAACGGTCGATGCAGGACCTTGTGGACATGGTG

CACCATCACCACCATGAGTTCGAGGAGCAGAAGCTGATCTCTGAGGAGGACCTGAACTAATAA
                                                                    1053
GTGGTAGTGGTGGTACTCAAGCTCCTCGTCTTCGACTAGAGACTCCTCCTGGACTTGATTATT
```

Table 19

Human monocyte chemotactic protein MCP-1 (9-76) sequence

ATGGTTACCTGCTGTTATAACTTCACCAACCGTAAAATCTCAGTGCAGAGGCTCGCGAGCTATAGAAGAATCACCAGCAGCAAGTGTCCC
TACCAATGGACGACAATATTGAAGTGGTTGGCATTTTAGAGTCACGTCTCCGAGCGCTCGATATCTTCTTAGTGGTCGTCGTTCACAGGG 90

AAAGAAGCTGTGATCTTCAAGACCATTGTGGCCAAGGAGATCTGTGCTGACCCCAAGCAGAAGTGGGTTCAGGATTCCATGGACCACCTG
TTTCTTCGACACTAGAAGTTCTGGTAACACCGGTTCCTCTAGACACGACTGGGGTTCGTCTTCACCCAAGTCCTAAGGTACCTGGTGGAC 18

GACAAGCAAACCCAAACTCCGAAGACTTGATGA 213
CTGTTCGTTTGGGTTTGAGGCTTCTGAACTACT

TABLE 20

A5B7 F(ab')$_2$sequences

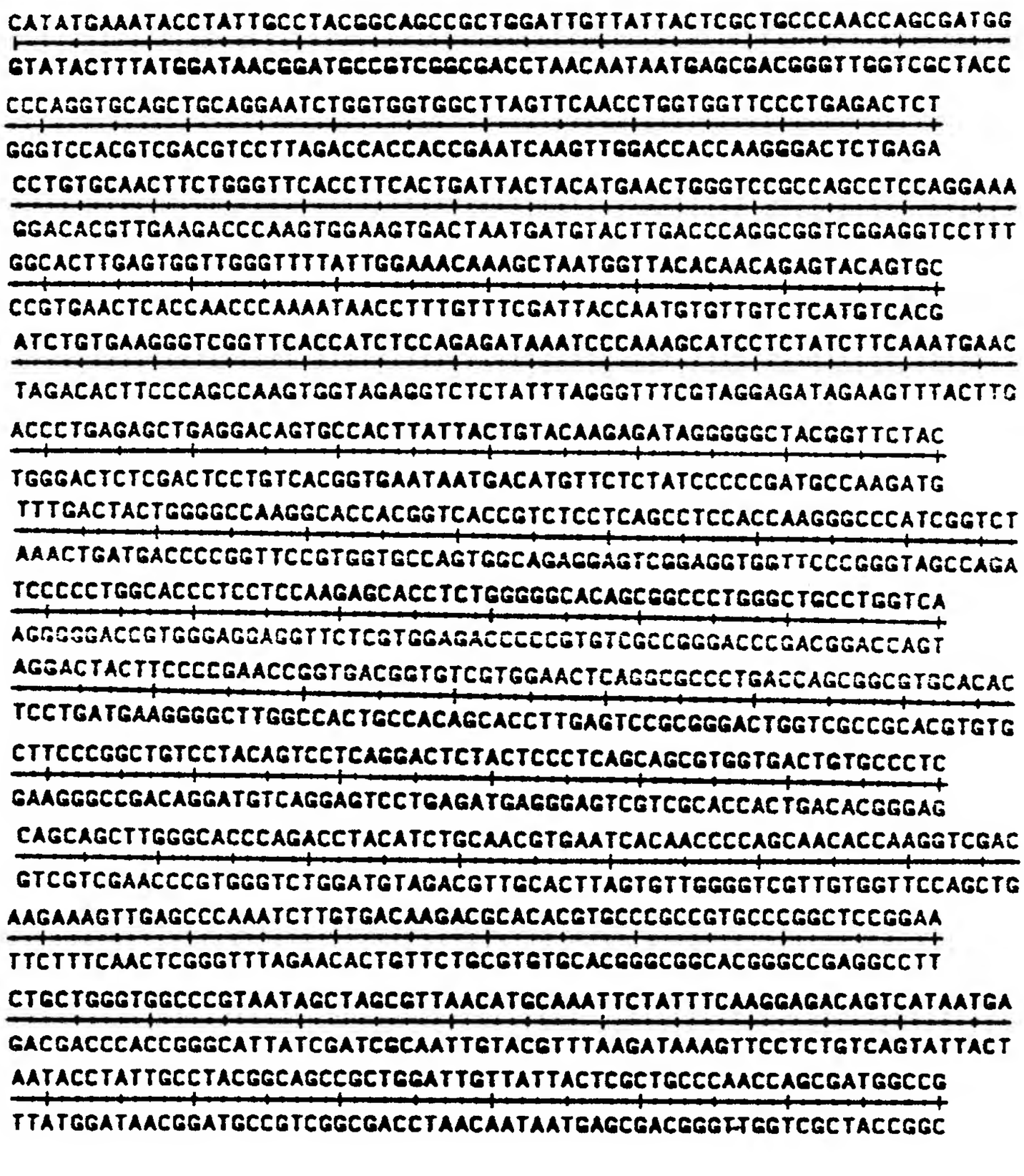
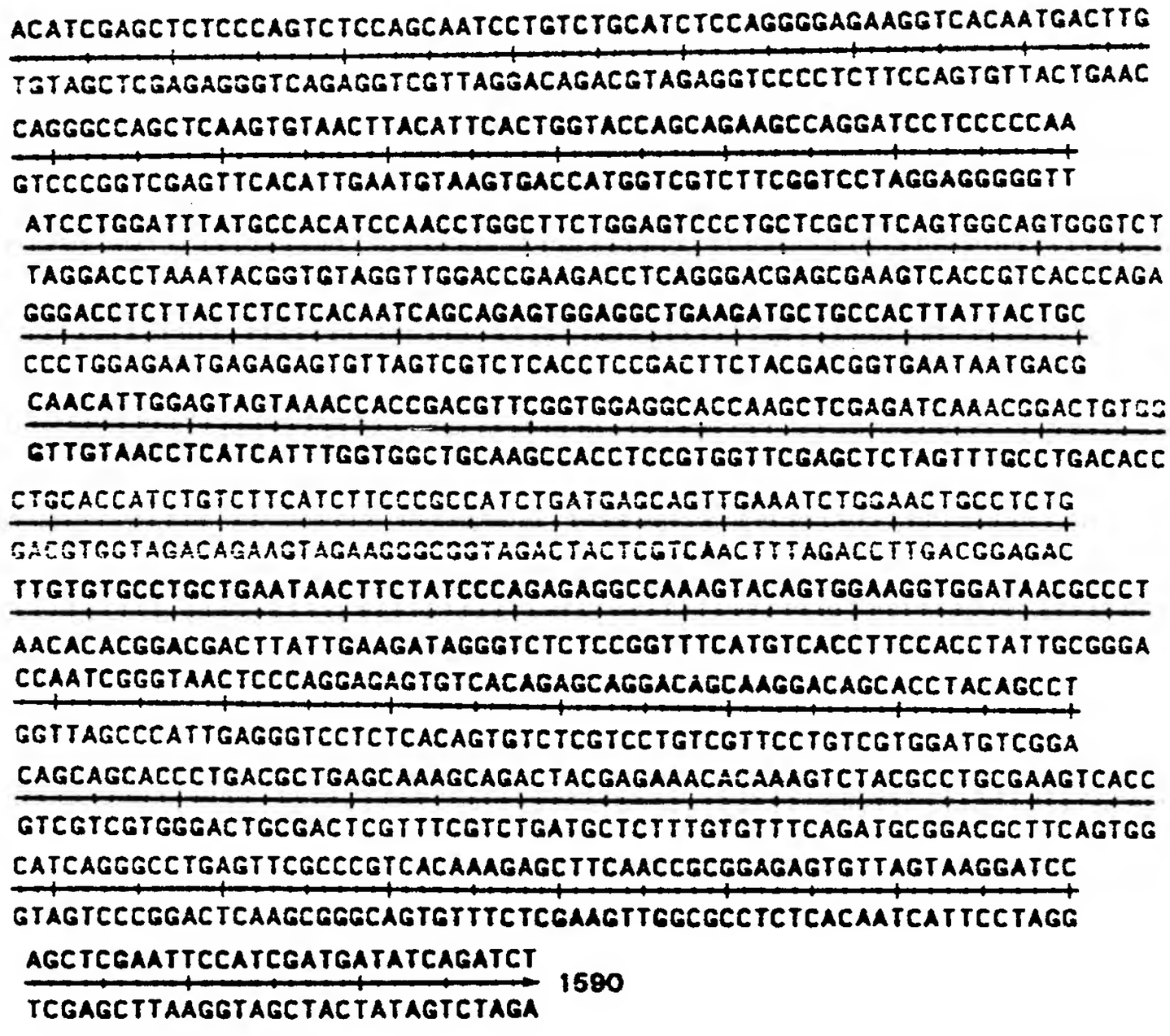


Table 20 /continued over

TABLE 20

A5B7 F(ab')$_2$sequences

Table 20 /continued



Claims:

1. A T7 based promoter-driven protein expression system comprising an operator sequence downstream of the T7 promoter sequence, and having a further operator sequence upstream of the T7 promoter sequence.

2. A protein expression system as claimed in claim 1 wherein the further operator sequence is a native lac operator (lacO) sequence.

3. A protein expression system as claimed in claim 1 wherein the further operator sequence is a perfect palindrome operator (ppop) sequence.

4. A protein expression system as claimed in claim 3 wherein the native operator sequence downstream of the T7 promoter sequence is replaced by a ppop sequence. so as to provide a tandem ppop operator.

5. A plasmid which comprises a target gene under T7 promoter control, and comprising an operator sequence downstream of the T7 promoter sequence, and having a further operator sequence upstream of the T7 promoter sequence.

6. A plasmid as claimed in claim 5 wherein the further operator sequence is a native lac operator (lacO) sequence.

7. A plasmid as claimed in claim 5 wherein wherein the further operator sequence is a perfect palindrome operator (ppop) sequence.

8. A plasmid as claimed in claim 4 wherein the native operator sequence downstream of the T7 promoter sequence is replaced by a ppop sequence, so as to provide a tandem ppop operator.

9. A plasmid selected from pZT7#3.0, pZT7#3.1, pZT7#3.2 and pZT7#3.3

10. The plasmid pZT7#3.3.

11. A host cell transformed by a plasmid as claimed in any one of claims 5-10

12. A host cell transformed by plasmid pZT7#3.3.

13. E. Coli transformed by pZT7#3.3.

14. A method of use of an expression system or host cells as claimed in any one of the previous claims in the production of recombinant protein.

15. Recombinant protein when produced by the method of claim 14.

Figure 1

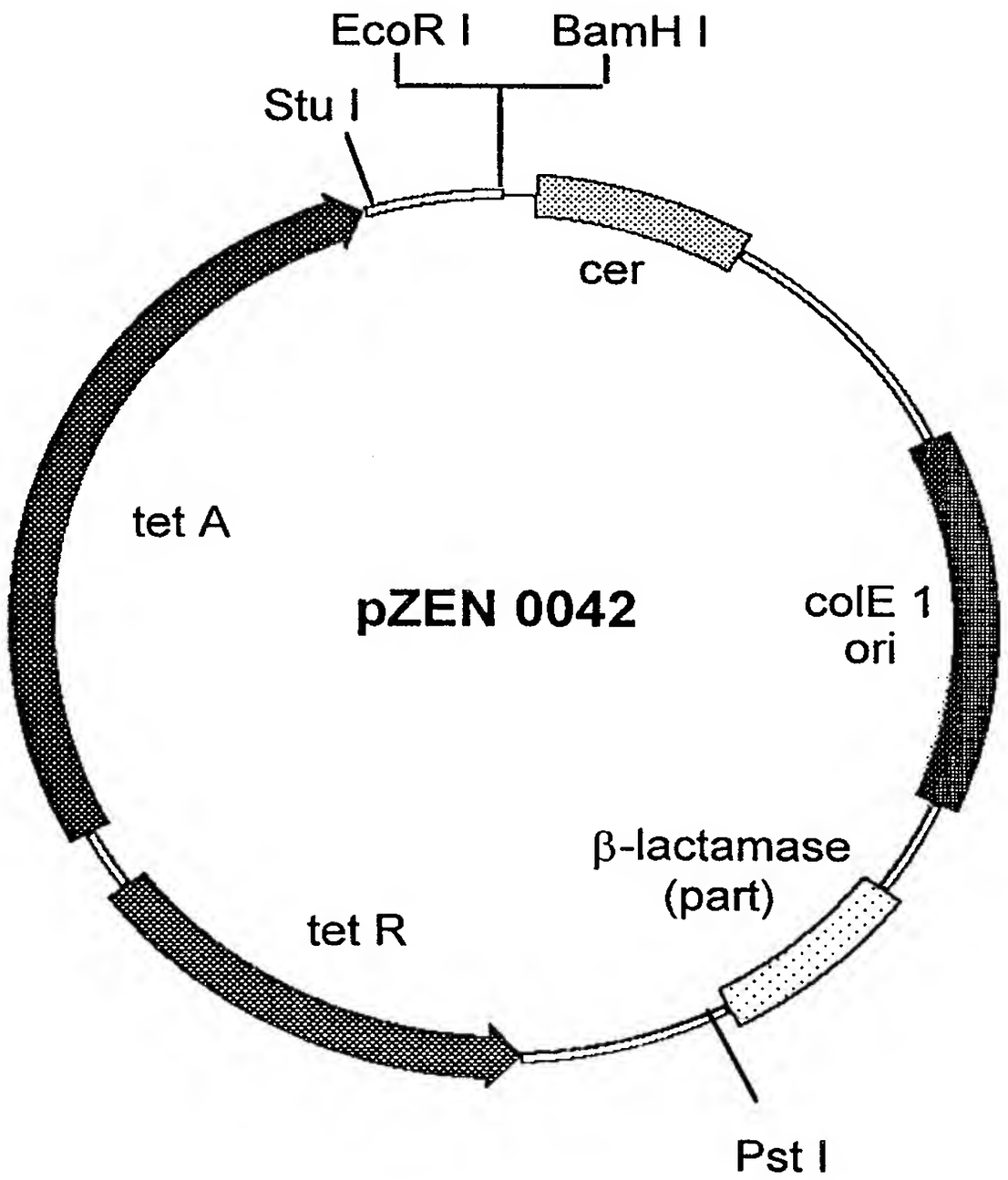
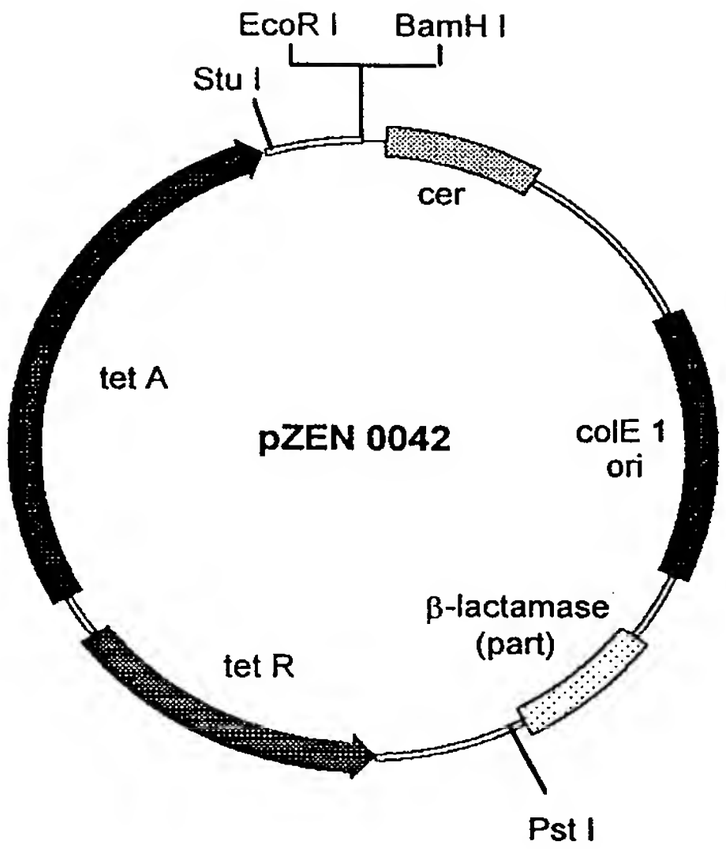
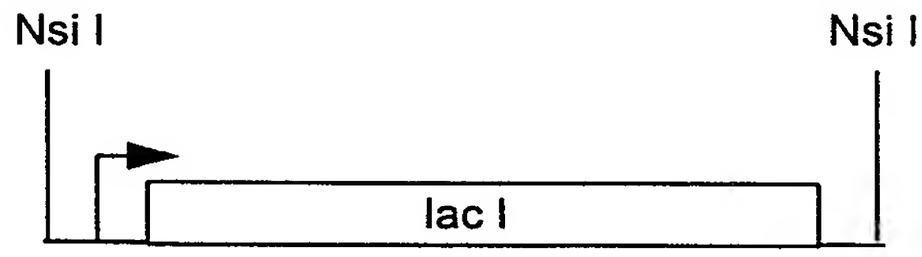

EcoR I
BamH I
Stu I
cer
tet A
pZEN 0042
colE 1
ori
β-lactamase
(part)
tet R
Pst I

Figure 2





Lac I sequence generated by PCR and cloned into Pst I site of pZEN 0042

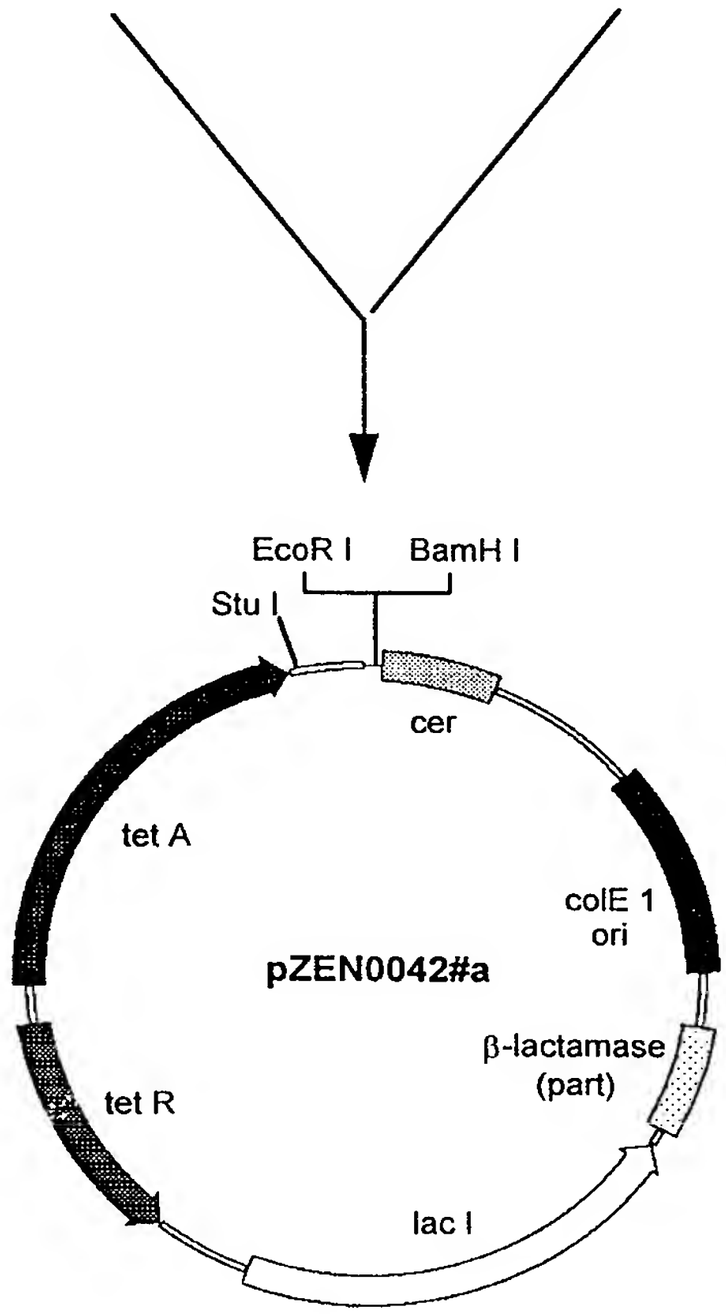


Figure 3

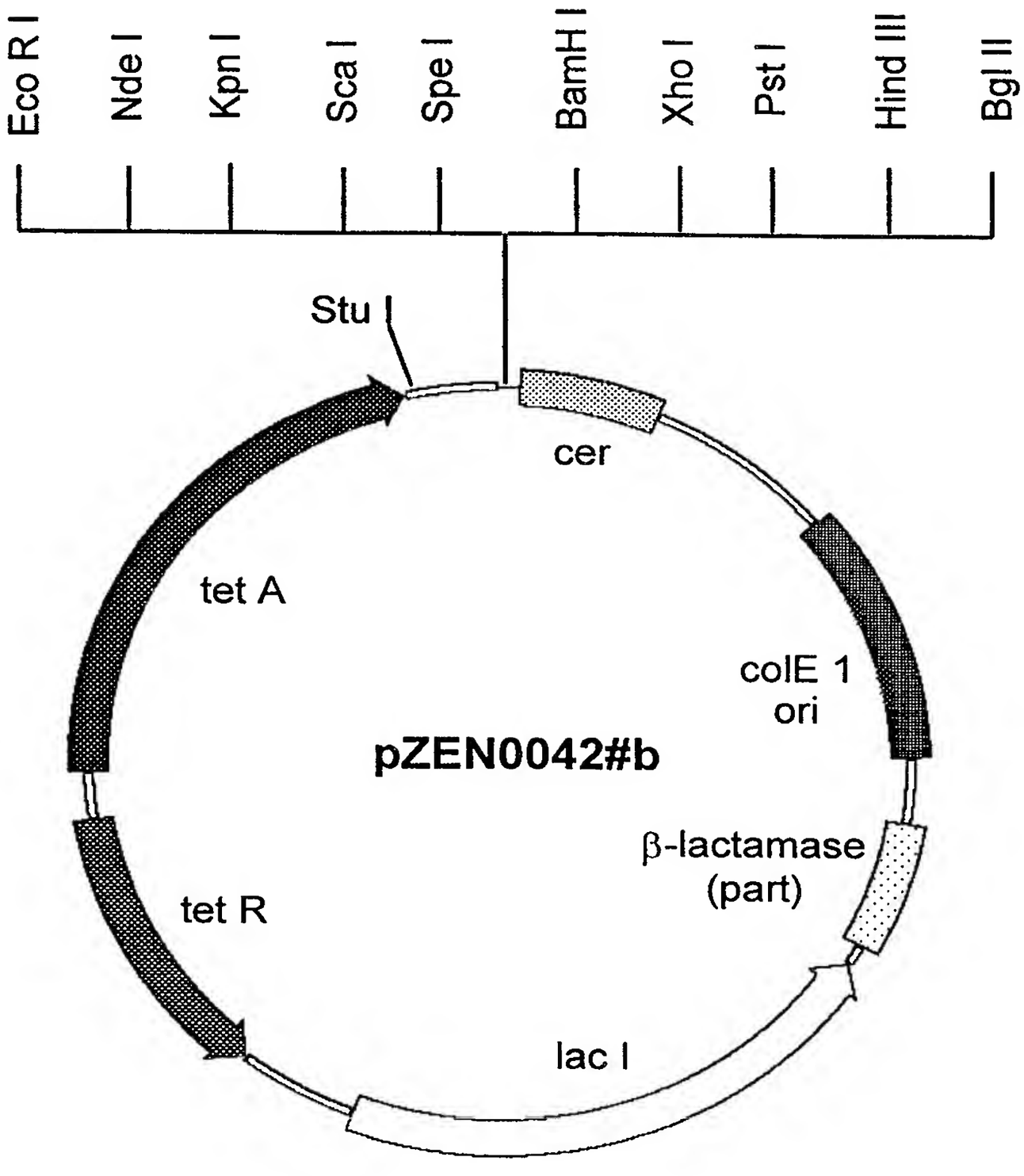

Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
Stu
cer
tet A
colE 1
ori
pZEN0042#b
β-lactamase
(part)
tet R
lac I

Figure 4

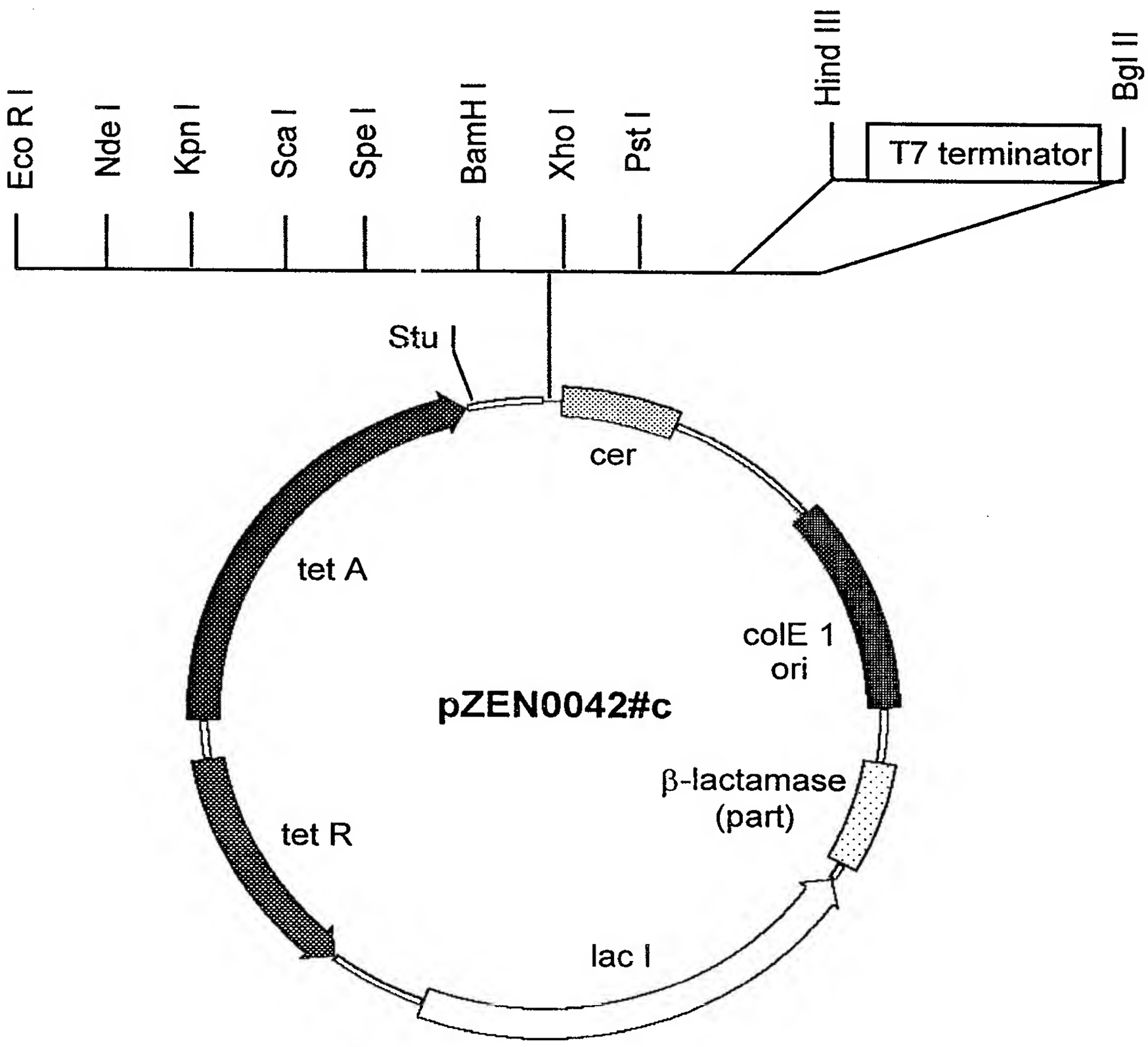
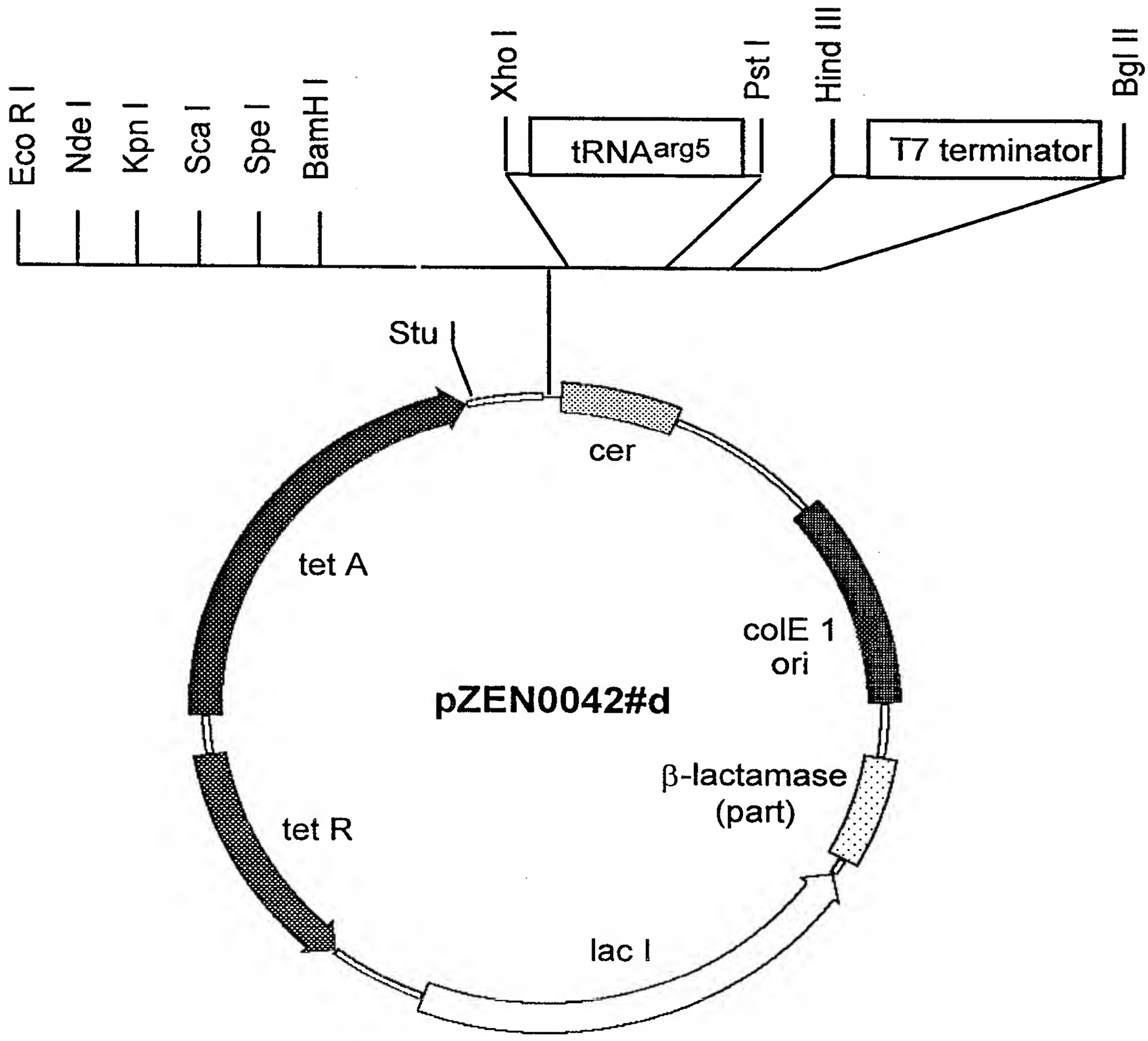

Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T7 terminator
Stu I
cer
tet A
colE 1
ori
pZEN0042#c
β-lactamase
(part)
tet R
lac I

Figure 5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Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
tRNAarg5
Pst I
Hind III
T7 terminator
Bgl II
Stu I
cer
tet A
colE 1
ori
pZEN0042#d
β-lactamase
(part)
tet R
lac I

Figure 6

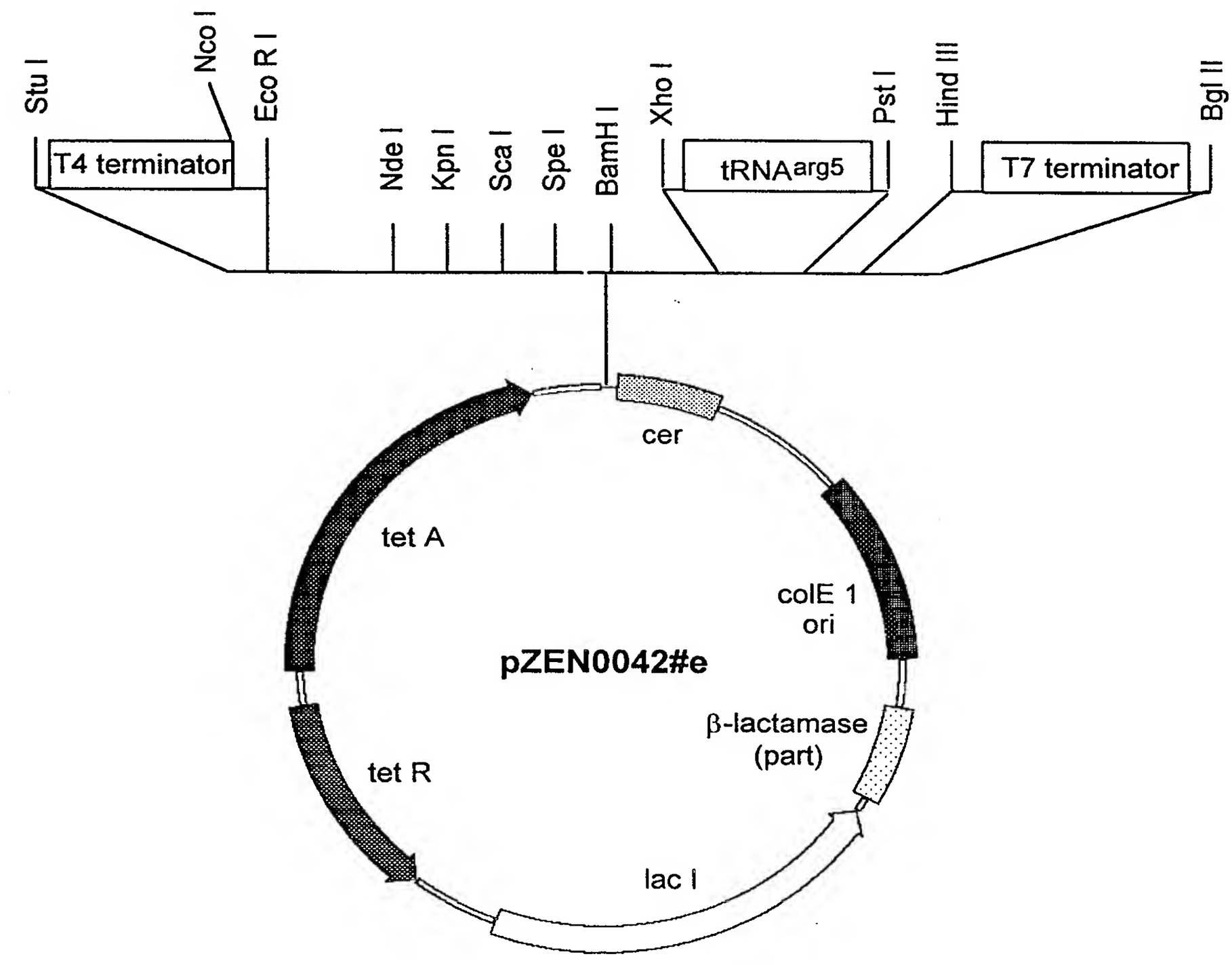

Stu I
Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
tRNAarg5
T7 terminator
cer
tet A
colE 1
ori
pZEN0042#e
β-lactamase
(part)
tet R
lac I

Figure 7

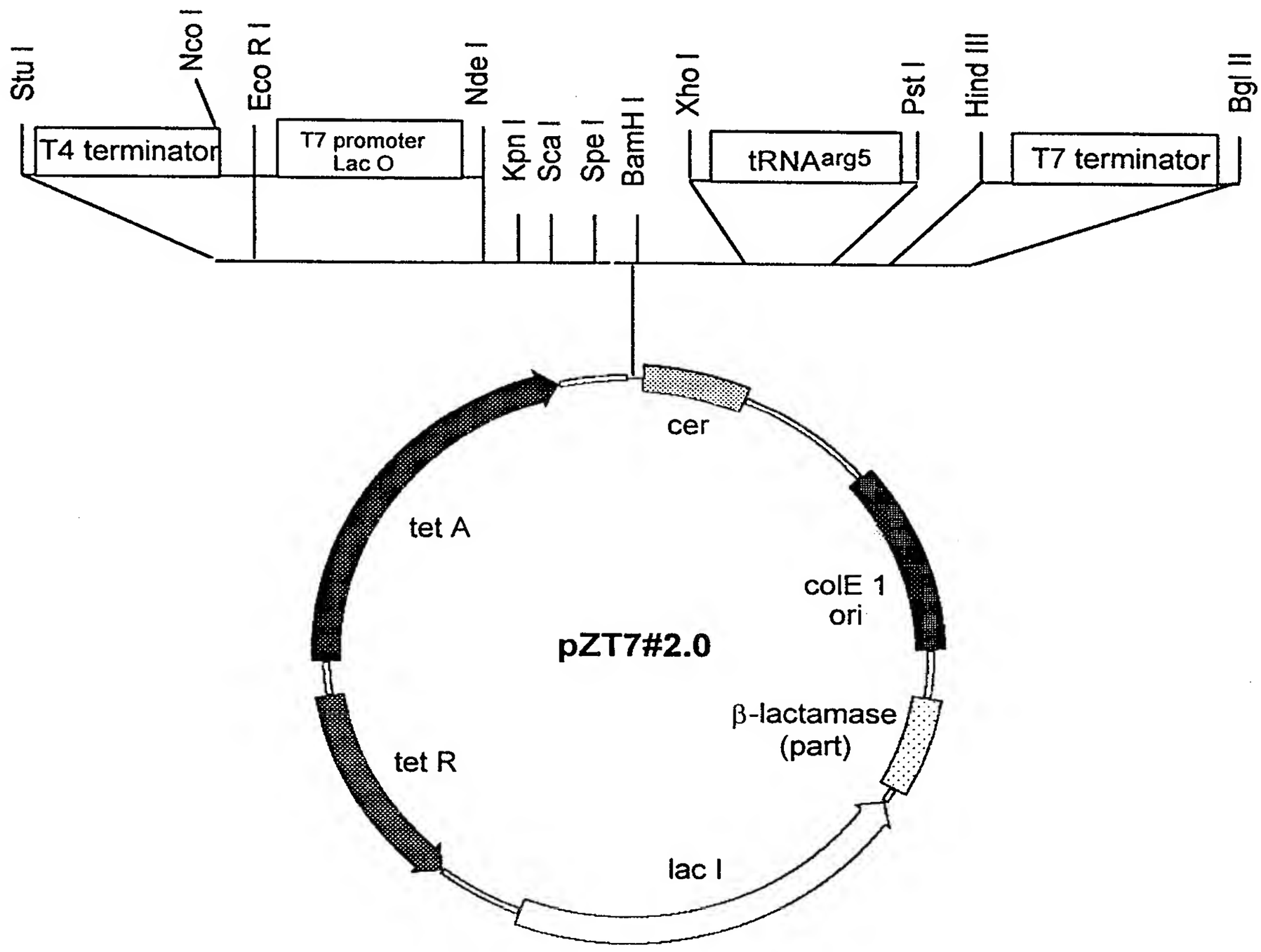

Stu I
Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
T7 promoter
Lac O
tRNAarg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#2.0
β-lactamase
(part)
tet R
lac I

Figure 8

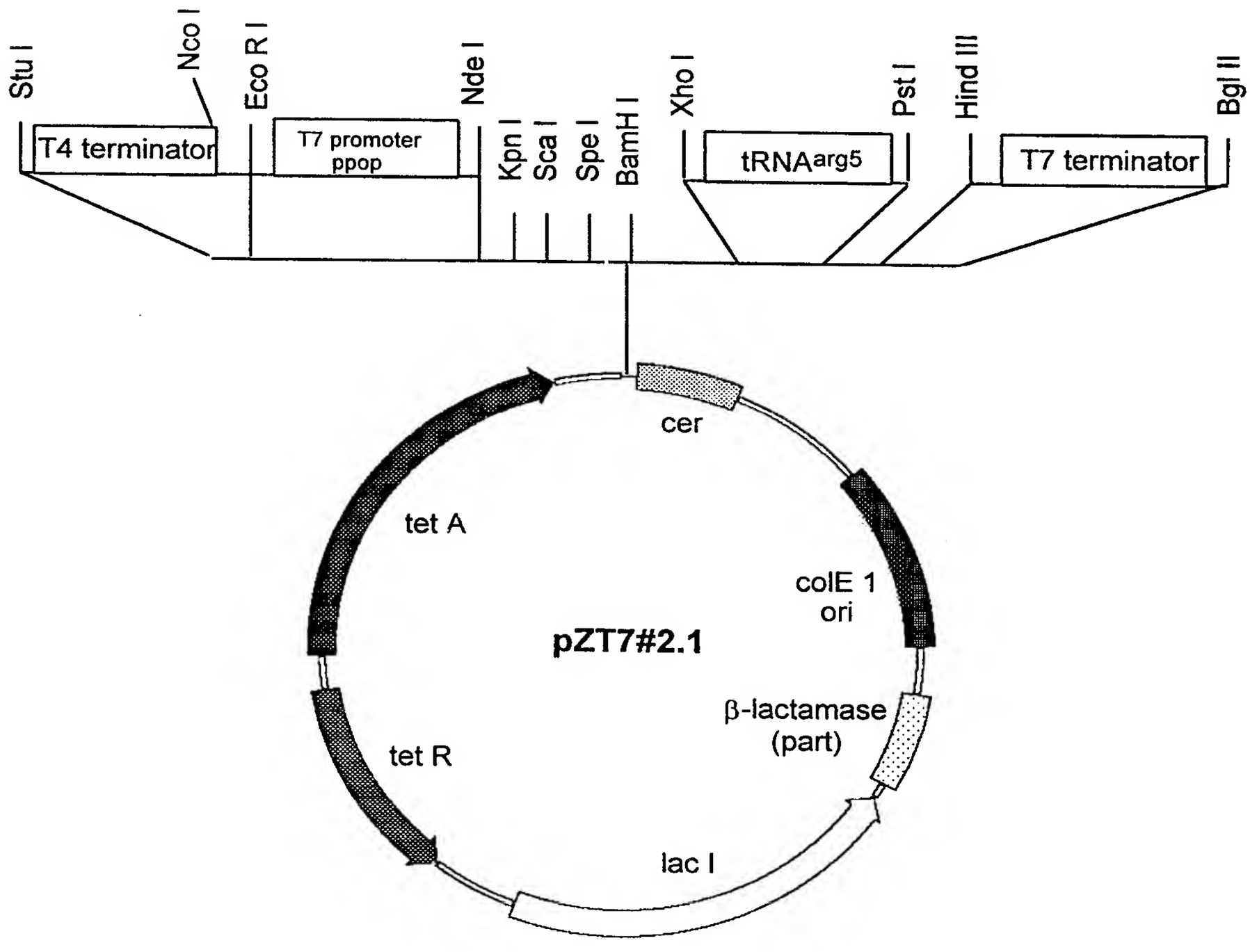

Stu I
Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
T7 promoter
ppop
tRNAarg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#2.1
β-lactamase
(part)
tet R
lac I

Figure 9

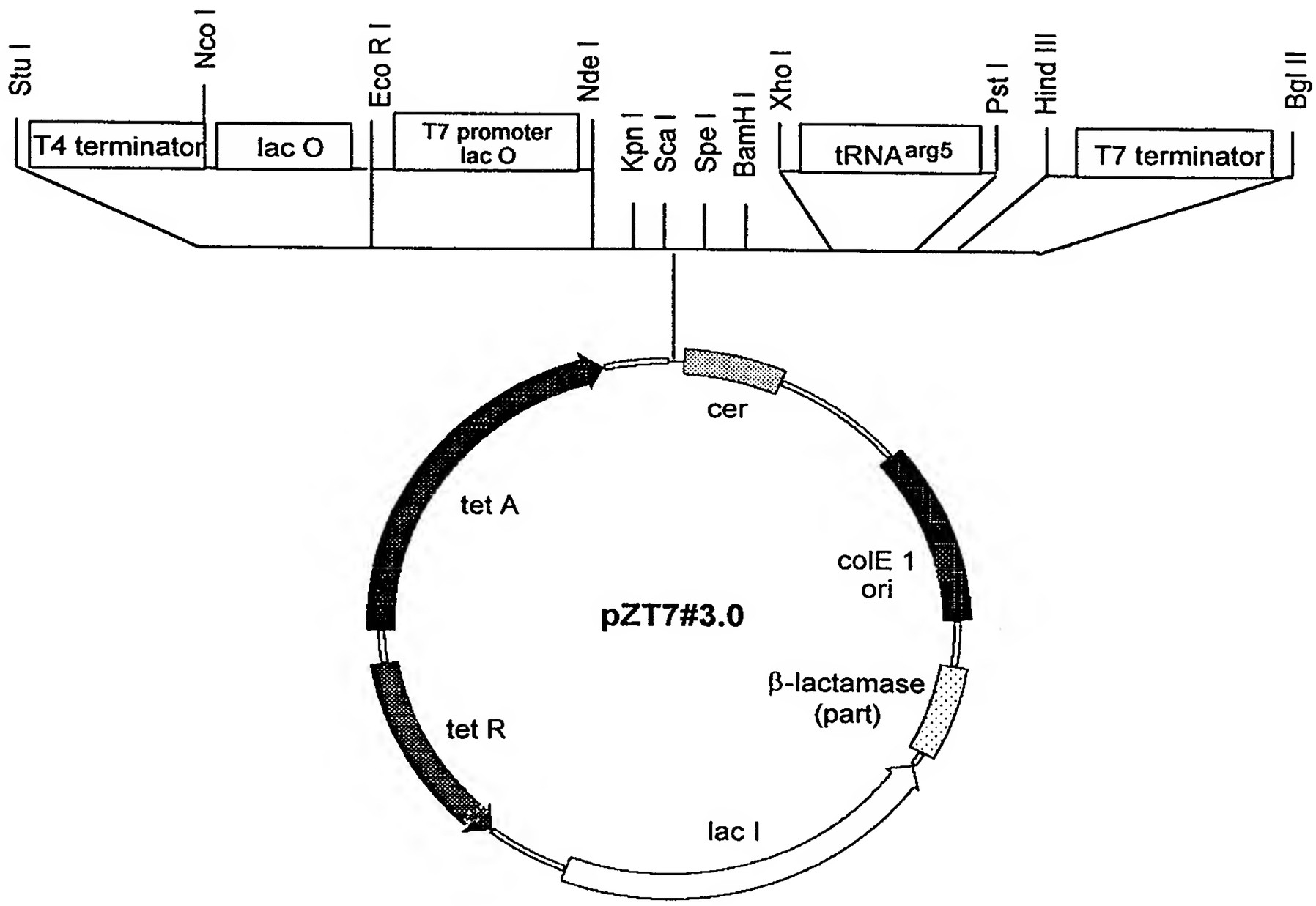
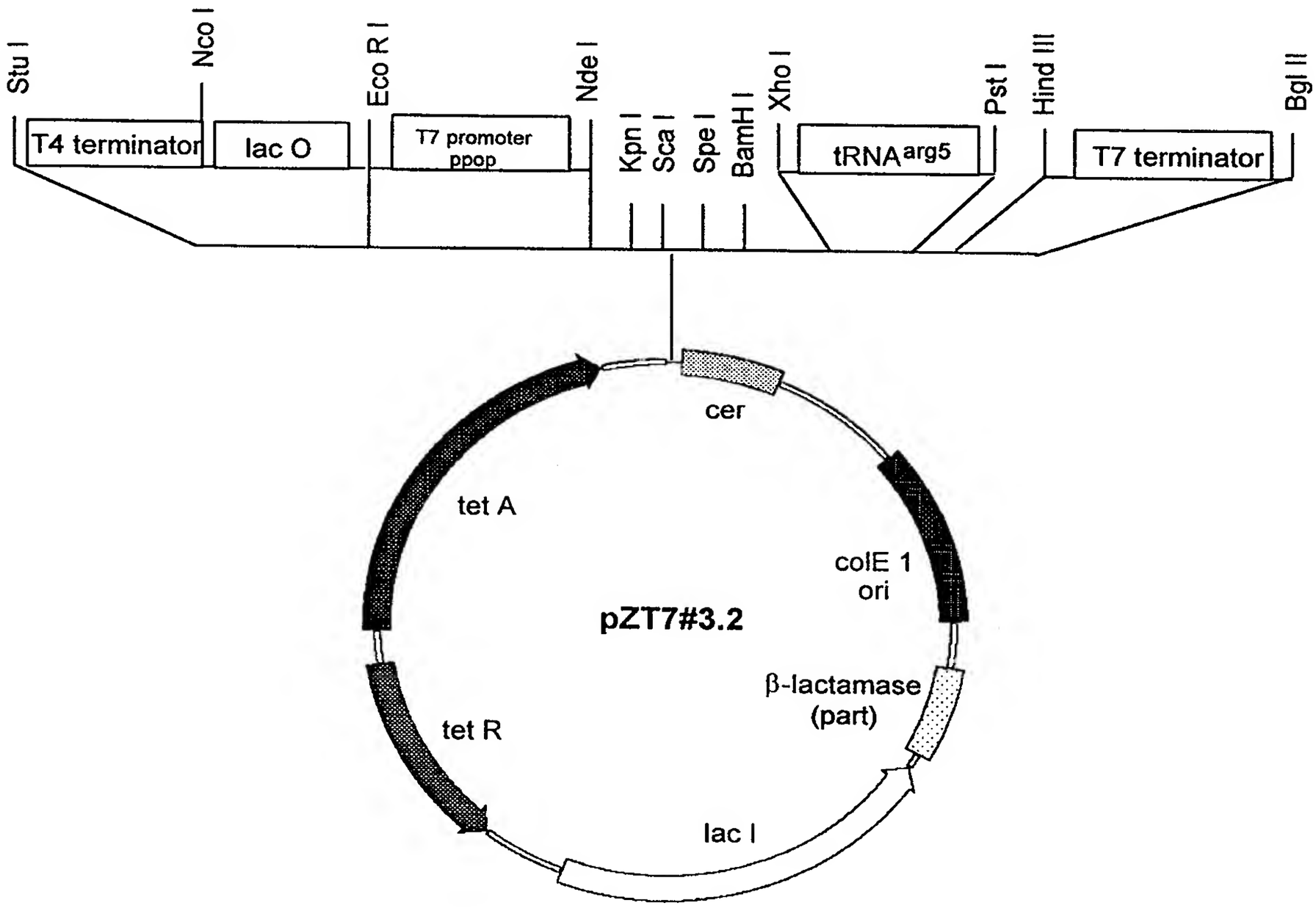

Stu I
Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
lac O
T7 promoter
lac O
tRNA arg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#3.0
β-lactamase
(part)
tet R
lac I

Figure 10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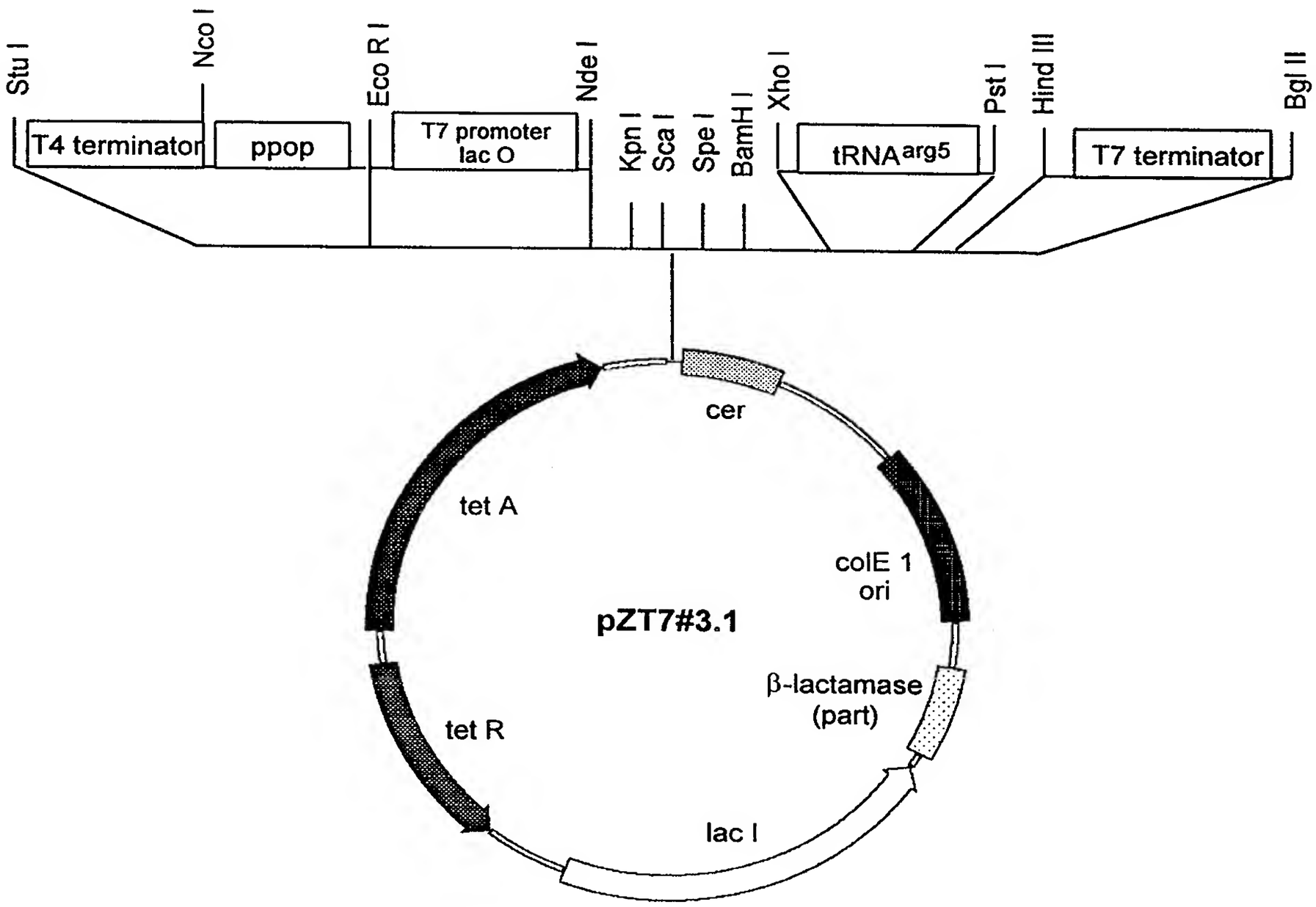
Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
lac O
T7 promoter
ppop
tRNA arg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#3.2
β-lactamase
(part)
tet R
lac I

Figure 11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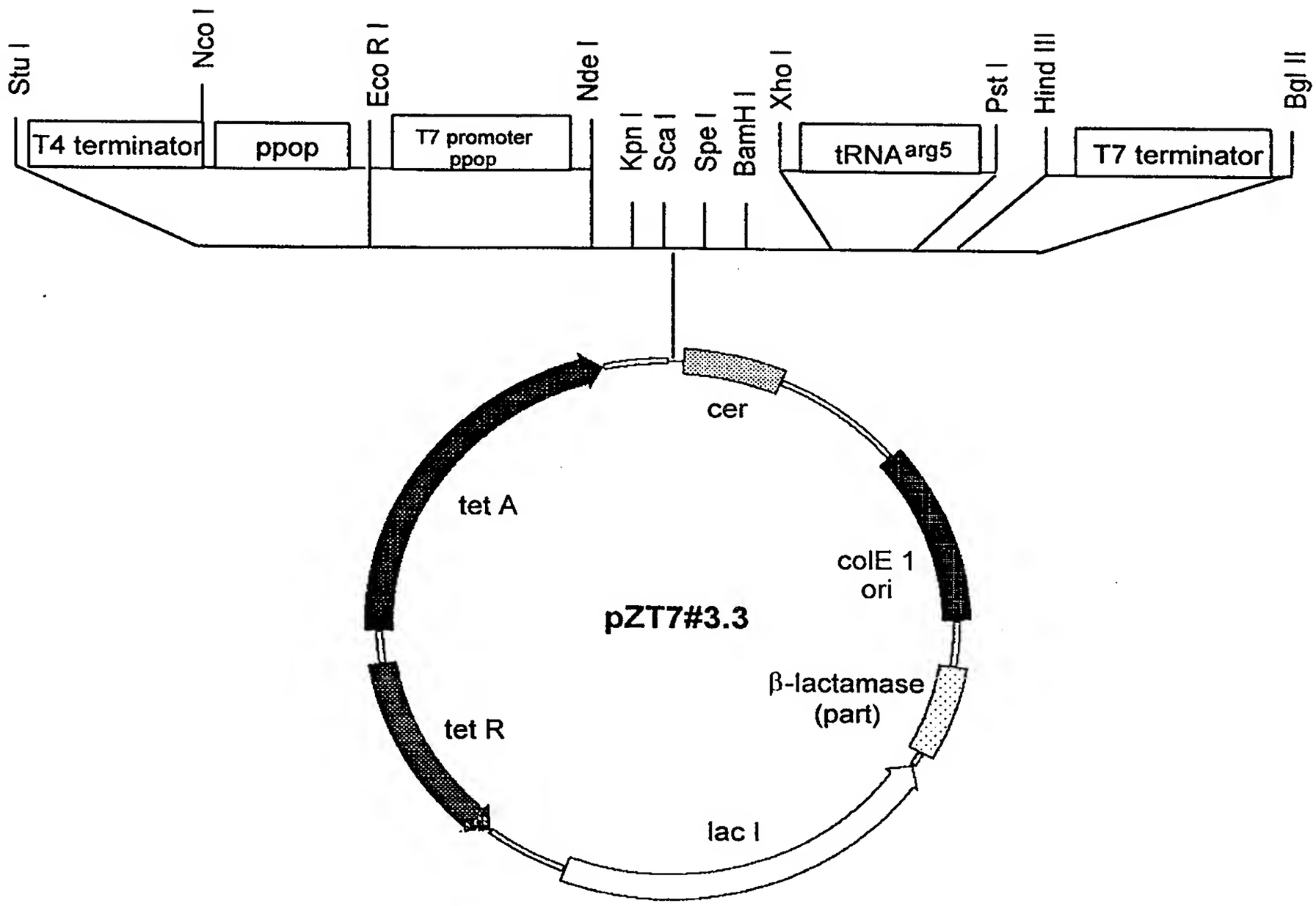
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
ppop
T7 promoter
lac O
tRNA arg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#3.1
β-lactamase
(part)
tet R
lac I

Figure 12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Nco I
Eco R I
Nde I
Kpn I
Sca I
Spe I
BamH I
Xho I
Pst I
Hind III
Bgl II
T4 terminator
ppop
T7 promoter
ppop
tRNA arg5
T7 terminator
cer
tet A
colE 1
ori
pZT7#3.3
β-lactamase
(part)
tet R
lac I

Figure 13

Q_p (max): mg/g/h

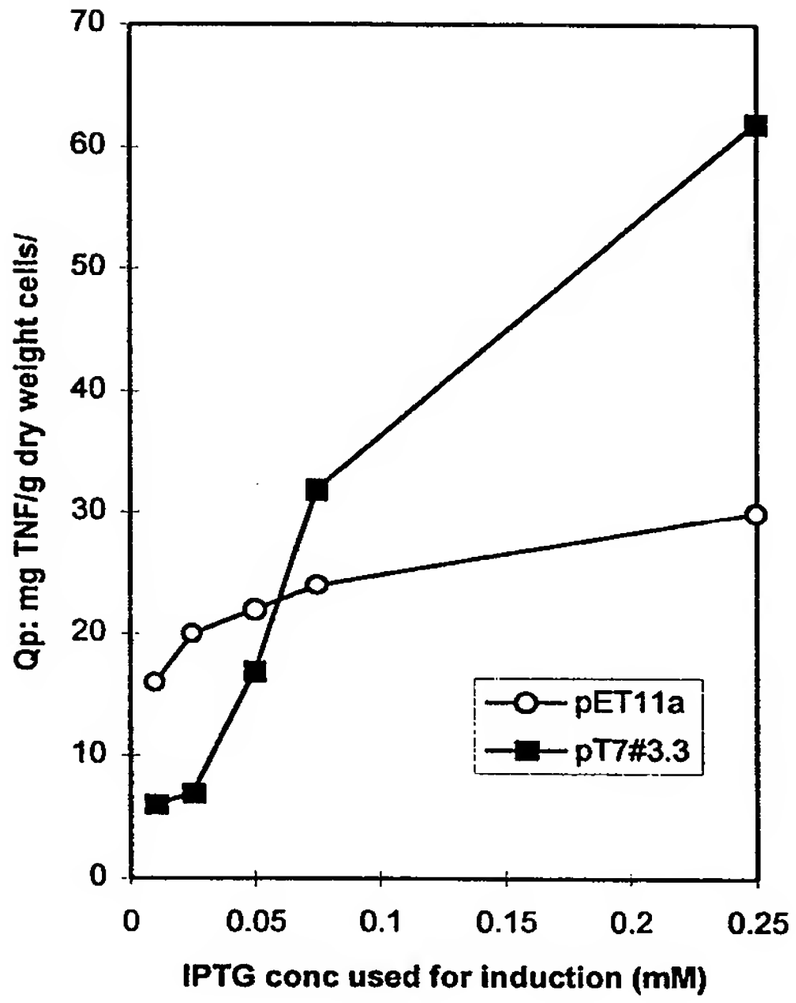


Figure 14

TNF accm (max)
% TMP

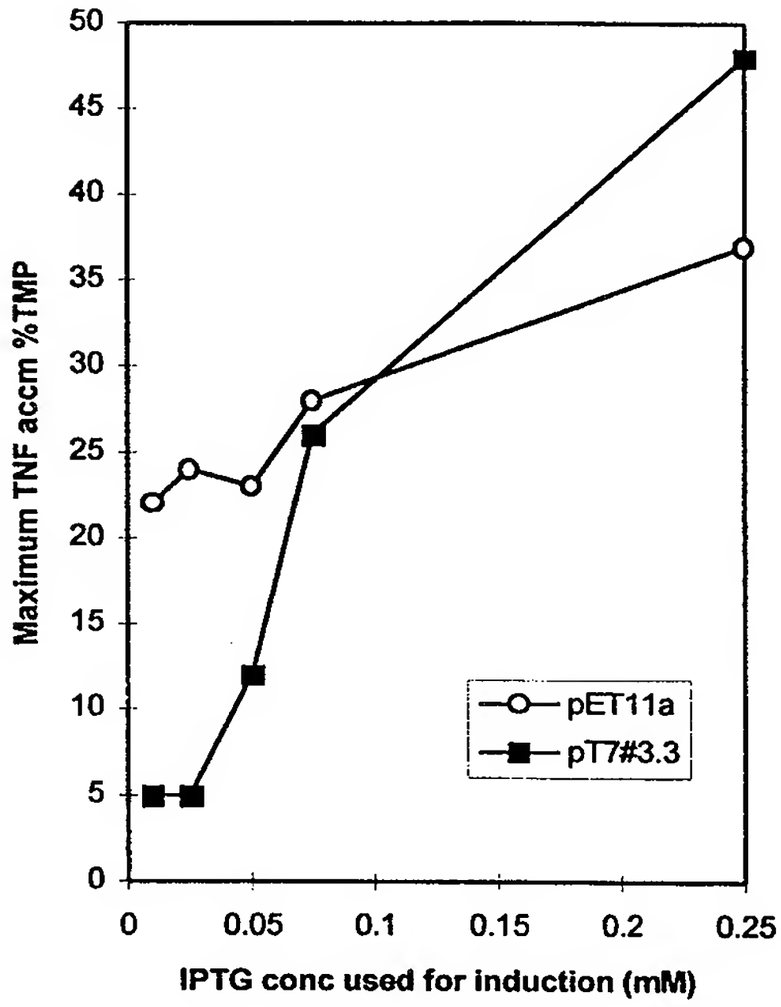


Figure 15

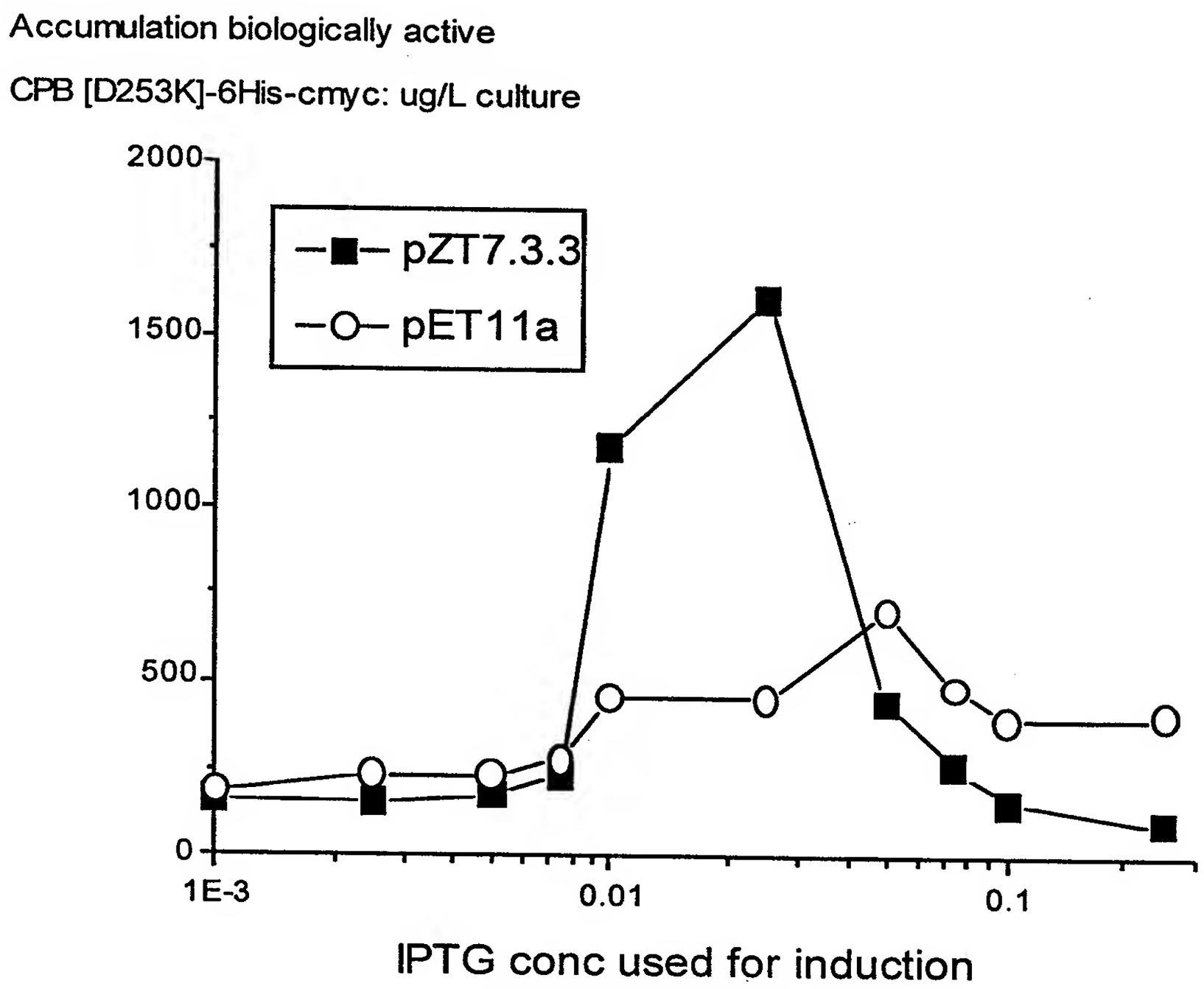
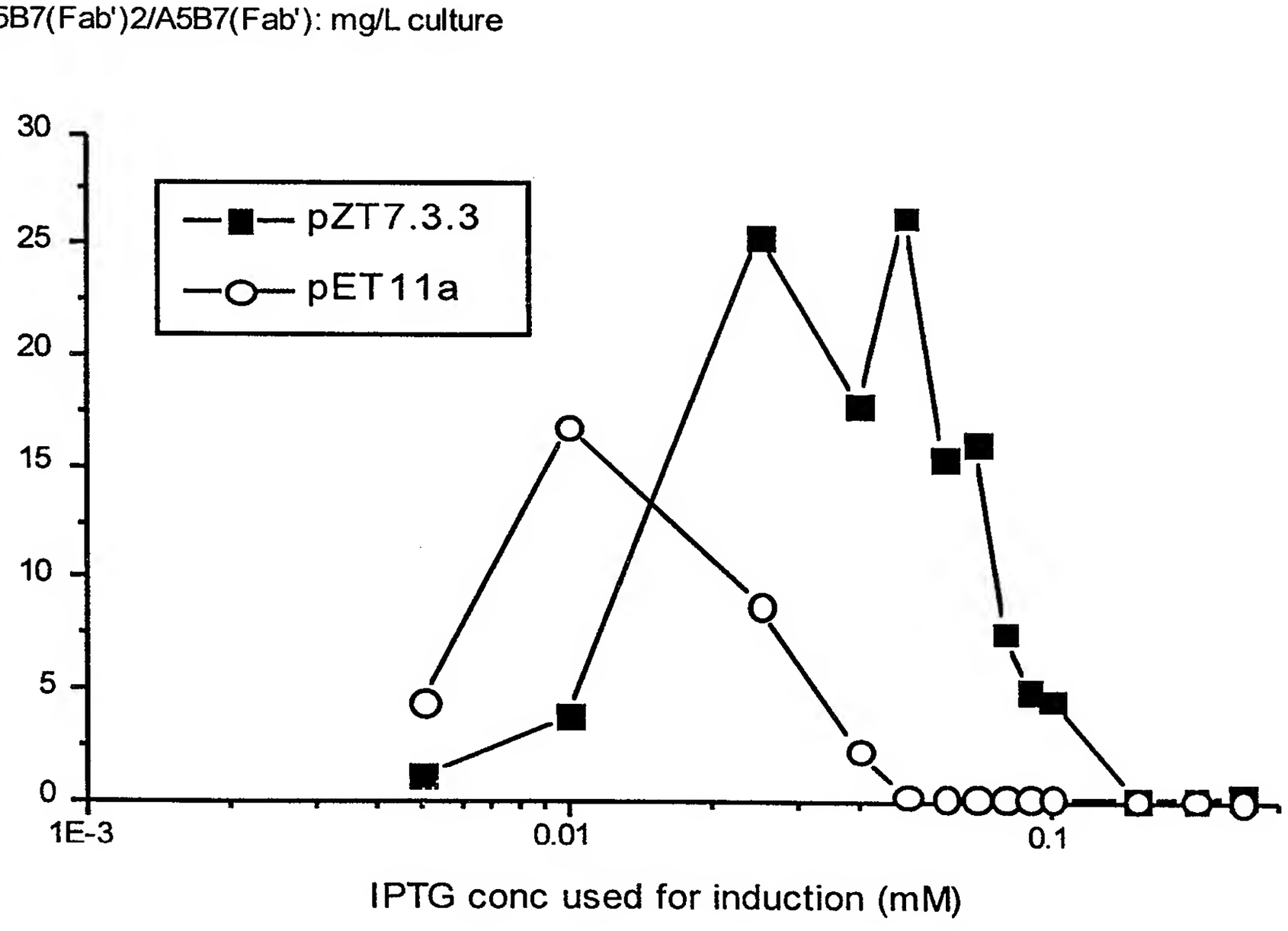

Accumulation biologically active
CPB [D253K]-6His-cmyc: ug/L culture
2000
1500
1000
500
0
pZT7.3.3
pET11a
1E-3
0.01
0.1
IPTG conc used for induction

Figure 16


A5B7(Fab')2/A5B7(Fab'): mg/L culture
30
25
20
15
10
5
0
pZT7.3.3
pET11a
1E-3
0.01
0.1
IPTG conc used for induction (mM)

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/02175

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/72 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUBENDORFF J W ET AL: "CONTROLLING BASAL EXPRESSION IN AN INDUCIBLE T7 EXPRESSION SYSTEM BY BLOCKING THE TARGET T7 PROMOTER WITH LAC REPRESSOR" JOURNAL OF MOLECULAR BIOLOGY, vol. 219, no. 1, 1991, pages 45-59, XP000605448 see the whole document	15
A	---	1-14
A	NOVAGEN: "pET EXPRESSION SYSTEM INFORMATION PACKAGE" August 1995 XP002084177 see the whole document	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1998	24/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/02175

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 502 637 A (ICI PLC) 9 September 1992 cited in the application see page 2, line 40 - line 45	1-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/GB 98/02175

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		CA 2061721 A	27-08-1992
		FI 920835 A	27-08-1992
		GB 2253852 A,B	23-09-1992
		JP 5056790 A	09-03-1993
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